REMARKS

After entry of the amendments above, claims 33-132, 145-304 and 367-446 will be pending. No new matter is added by the amendments to the claims. Applicants respectfully request reconsideration and withdrawal of the outstanding rejections in view of the following remarks.

1. ***Formal Matters***

Information Disclosure Statement

On page 2 of Paper No. 28, paragraphs A, C and D, the Examiner indicates that "since Statutory Declarations are not proper subject matter for an IDS, Applicants have not submitted a Form PTO-1449" for the Statutory Declarations submitted in the Information Disclosure Statements mailed April 18, 2002, January 23, 2002, and December 21, 2001.

Applicants wish to thank Examiner Landsman for clarifying the significance of the Examiner's comments in paragraphs A, C and D in a telephonic interview with Applicants' representative, Melissa Pytel, on January 29, 2003. Because Applicants wish to have the Statutory Declarations considered by the Examiner and marked in the record as such, Applicants provide herewith an Information Disclosure Statement and Form SB/08, listing the Statutory Declarations.

As discussed in the telephonic interview with Examiner Landsman, Statutory Declarations are proper material for an Information Disclosure Statement. 37 C.F.R. §1.56(a) states that an applicant has a duty to disclose to the Patent Office all information that is material to patentability. 37 C.F.R. §1.98(a)(1) states that an information disclosure statement shall include a list and a legible copy "of all patents, publications, applications, *or other information* submitted for consideration by the Office." (Emphasis added.) Applicants submit that the Statutory Declarations constitute "other information" under 37 C.F.R. §1.98 that may include information that is material to the patentability of the instant application and are therefore contemplated by 37 C.F.R. §1.56(a) as information to be included in an IDS. Applicants therefore respectfully request that the Statutory Declarations listed on the form SB/08 be initialed as considered by the Examiner.

Withdrawal of Method Claims

The Examiner has withdrawn claims 45-52, 65-72, 85-92, 105-112, 125-132, 157-164, 177-184, 197-204, 217-224, 237-244, 257-264, 277-284, 297-304, 318-325, 339-349, 359-366, 379-386, 399-406, 419-426 and 439-446 as non-elected method claims (Paper No. 28, page 2, paragraph H)

Applicants respectfully request that the withdrawn method claims be rejoined upon notification of allowable product claims. *See,* MPEP § 821.04 (Where an applicant is required to elect either product or process claims that are presented in the same application, the claims to the non-elected invention will be withdrawn from further consideration under 37 CFR 1.142 and rejoined when a product claim is subsequently found allowable).

2. Information Disclosure Statement

On page 3 of Paper No. 28, the Examiner indicates that reference EY, an International Search Report, submitted March 12, 2002, has been lined through because "an international search report is not a proper reference for an IDS."

Applicants respectfully submit that the International Search Report was properly submitted to show the characterization of the references cited by the Examiner during prosecution of the corresponding PCT application. As discussed in connection with the Statutory Declarations above, Applicants submit that the International Search Report constitutes "other information" under 37 C.F.R. §1.98 that is material to patentability. However, since all of the references cited in the Search Report were listed on the form 1449 and initialed as considered by the Examiner, the issue is moot.

3. Double Patenting Rejections

Provisional Double Patenting Rejection

The Examiner *provisionally* rejected pending claims 33-44, 53-64, 73-84, 93-104, 113-124, 133-156, 165-176, 185-196, 205-216, 225-236, 245-256, 265-276, 285-296, 305-317, 326-338, 347-358, 367-378, 387-398, 407-418, and 427-438 under the judicially created doctrine of obviousness-type double patenting over the claimed invention in the following copending U.S. Applications: 09/257,272, 09/935,726, 09/623,725, and 09/107,997.

Applicants acknowledge the provisional rejection. Upon receipt of a notice of allowance in this or in one of the above-referenced applications, Applicants will file an appropriate

disclaimer in the remaining application, to the extent that such a disclaimer is necessary, or will cancel any conflicting claims that remain pending.

Possible Provisional Double Patenting Rejections

The Examiner also indicated the claims of the instant application *may* receive a provisional rejection for double patenting in a subsequent Office Action over U.S. Application Nos. 10/060,523, 10/084,488, 10/127,551, 10/120,398, 10/120,377, and 10/120,414. At the time the present Office Action was mailed, the applications were either not available to the Examiner or no election had been made. Applicants thank the Examiner for the notification that a future provisional rejection may be issued for double patenting over the listed applications.

4. Enablement Rejections Under 35 U.S.C. §112, First Paragraph

A. ATCC Deposit

The Examiner has rejected claims 73-84, 93-104, 113-124, 245-256, 285-296, and 326-338 under 35 U.S.C. §112, first paragraph, as containing subject matter which is not enabled because there is no indication in the specification as to the public availability of the deposited nucleic acid molecules contained in ATCC Deposit Nos. 75698 and 97149 (Paper No. 28, page 7).

In response, Applicants' representative hereby gives the following assurance by signature below:

Human Genome Sciences, Inc., the assignee of the present application, has deposited biological material under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure with the following International Depository Authority: American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209. This deposit comprises cDNA sequences encoding Vascular Endothelial Growth Factor-2. The deposits were given ATCC Accession Numbers 75698 and 97149 and were made on March 4, 1994 and May 12, 1995, respectively.

In accordance with M.P.E.P. §2410.01 and 37 C.F.R. §1.808, assurance is hereby given that all restrictions on the availability to the public of ATCC Accession Numbers 75698 and 97149 have been removed due to the issuance of U.S. patents on priority applications. A copy of

the ATCC Deposit Receipt for Accession Numbers 75698 and 97149 are enclosed herewith as **Attachments A and B**, respectively.

In light of the above statement regarding availability of the deposited material, Applicants submit that the Examiner's rejection is obviated and respectfully request withdrawal of the rejection.

B. Fragments

The Examiner rejected claims 133-156, 165-176, 225-236, 245-256, 265-276, 285-296, 305-317, 326-338, 407-418, and 427-438 under 35 U.S.C. §112, first paragraph, as not enabled. Applicants respectfully disagree and traverse the rejection.

The test for enablement under 35 U.S.C. §112, first paragraph is "whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." *United States v. Telectronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988). Undue experimentation is experimentation that requires a level of ingenuity beyond that expected from one of ordinary skill in the field. *Fields v. Conover*, 443 F2d 1386, 1390 (CCPA 1971). The factors that can be considered in determining whether an amount of experimentation is undue have been set forth in *In re Wands*, 858 F2d 731, 737 (Fed. Cir. 1988). Among these factors are: the amount of effort involved, the guidance provided by the specification, the presence of working examples, the amount of pertinent literature and the level of skill in the art. The test for undue experimentation is not merely quantitative. A considerable amount of experimentation is permissible if it is merely routine. *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1376, 1384 (Fed. Cir. 1986). Furthermore, Applicants respectfully point out that compliance with the enablement requirement does not turn on whether or not an example is disclosed. M.P.E.P § 2164.02.

Applicants respectfully submit that one skilled in the art could make and use any and all polypeptides encompassed by the claims without undue experimentation. Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of the instant claims under 37 U.S.C. §112, first paragraph for lack of enablement.

i. Specific Fragments

Applicants respectfully submit that claims 145-156 and 165-176, which are directed towards specific fragments of SEQ ID NO:2 (*i.e.*, amino acids 71 to 396; and amino acids 24 to

396) are enabled by the specification. With respect to claims 133-144, Applicants maintain that the claims are enabled. However, in the interest of advancing prosecution on the merits, claims 133-144 have been canceled without prejudice or disclaimer.

Independent claims 145 and 165 both recite fragments having a specific sequence of amino acids (*i.e.,* amino acids 71 to 396 and amino acids 24 to 396, respectively, of SEQ ID NO:2). Applicants respectfully submit that one of skill in the art can easily make polypeptides having a specific sequence.

Applicants further submit that one of skill in the art would know how to use the claimed fragments.

a. The Specification Teaches the Significance of Retaining the Structural Motif Spanning Amino Acid Residues 108-188 For Maintaining VEGF-2 Biological Activity

The specification teaches that amino acids 108-188 correspond to a region having a structural motif characterized by eight cysteine residues (*See,* SEQ ID NO:2 and FIG.3). The specification clearly teaches the significance of this structural motif for the retention of biological activity. Furthermore, at the time the application was filed, it was known to those of skill in the art that the members of the PDGF/VEGF family of growth factors share a strictly conserved structural motif, i.e., a region spanning eight strictly conserved cysteine residues (see specification at page 2, lines 15-17 and page 9, lines 11-12). The specification characterizes VEGF-2 as a new member of the PDGF/VEGF family that shares this strictly conserved motif. In particular, FIG. 3 illustrates the amino acid homology between the members of the PDGF/VEGF family and highlights the location of the eight strictly conserved cysteine residues. The specification indicates that, in addition to the conserved eight cysteine residues, a signature consensus sequence for the PDGF/VEGF family, PXCVXXXRCXGCCN, is also conserved in VEGF-2 (specification at page 9, lines 12-14). This conserved fourteen amino acid region is contained within the region spanned by the eight strictly conserved cysteine residues and corresponds to amino acid residues 131 to 144 of VEGF-2 (as shown in SEQ ID NO:2.).

The instant specification also teaches that the conserved motif is important for retention of biological activity. Applicants direct the Examiner's attention to the specification at page 9, lines 21-25 describing active fragments as "any portions of the full length amino acid sequence which have less than the full 419 amino acids of the full length amino acid sequence as shown in

SEQ ID NO: 2, but still contain the eight cysteine residues shown conserved in Figure 3 and that still have VEGF2 activity." Indeed, Applicants submit that each time the specification describes fragments that contain the eight conserved cysteine residues, such fragments are concurrently described as retaining biological activity, *see, e.g.*, specification at page 9, lines 21-25. Therefore, upon reading the specification, one skilled in the art would recognize that the eight conserved cysteine residues are important for biological activity.

Moreover, Applicants submit that the specification not only provides sufficient guidance as to the structure, i.e., the region of SEQ ID NO:2 containing the eight conserved cysteine residues that is capable of retaining biological activity, but also provides sufficient guidance as to assays and methods for evaluating whether a polypeptide having less than the full length sequence of VEGF2 would retain biological activity. *See, e.g.*, specification, Examples 5 and 6.

The fact that some experimentation may be necessary to determine whether a polypeptide fragment retains biological activity does not necessarily make it undue, if the art typically engages in such experimentation. *In re Certain Limited-Charge Cell Culture Microcarriers,* 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd sub nom., Massachusetts Institute of Technology v. A.B. Fortia,* 774 F.2d 1104 (Fed. Cir. 1985). *See also, Hybritech, Inc. v. Monoclonal Antobodies, Inc.*, 802 F.2d 1376, 1384 (Fed. Cir. 1986). "The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." M.P.E.P. §2164.06 (*citing, In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988)). Furthermore, under 35 U.S.C. § 112, an inventor is not required to disclose a test of <u>every</u> species encompassed by their claims," even in an unpredictable art. *In re Angstadt*, 190 USPQ 214, 218 (CCPA 1976) (emphasis in original). Finally, enablement is not precluded if some embodiments of the claimed invention are inoperative. Indeed, the M.P.E.P. states that "[t]he presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled." *See,* M.P.E.P. § 2106.08(b).

Both independent claims 145 and 165 recite fragments that include the conserved eight cysteine residue motif and the signature fourteen amino acid consensus sequence PXCVXXXRCXGCCN. Thus, one of skill would reasonably expect the claimed fragments to retain biological activity. Furthermore, one skilled in the art could readily determine whether or not the claimed polypeptides comprising amino acid residues 71 to 396 of SEQ ID NO:2 (claim

145) or amino acid residues 24 to 396 of SEQ ID NO:2 (claim 165) retain biological activity, using the assays and methods described in the specification and others known in the art, without undue experimentation.

b. One Skilled In the Art Would Reasonably Expect that Amino Acid Residues 108-188 would be Important for Biological Activity

Based on the teachings of the specification, one skilled in the art would recognize the significance of retaining the structural motif spanning amino acid residues 108-188 for maintaining biological activity. Applicants respectfully submit that one skilled in the art would also reasonably expect that polypeptides comprising amino acid residues 71 to 396 of SEQ ID NO:2 (claim 145) or amino acid residues 24 to 396 of SEQ ID NO:2 (claim 165), which include the structural motif, would retain biological activity.

The Declaration of Dr. Stuart Aaronson ("Aaronson Declaration I"), which was submitted in co-pending U.S. Patent Application Serial No. 08/465,968, appended hereto as **Attachment C**, demonstrates that one skilled in the art could reasonably expect that a polypeptide containing at least the highly conserved structural motif would retain biological activity based on comparative sequence analysis of VEGF-2 and known members of the PDGF/VEGF family, as provided by the specification. Applicants wish to point out that the numbering of the amino acid residues in Aaronson Declaration I is different from the residue number in the present application. Amino acid residues 85-186, as discussed in Aaronson Declaration I, correspond to amino acid residues 108-188 in the present application.

Aaronson Declaration I demonstrates that, at the time the instant application was filed, it was known by those of skill in the art that the retention of conserved structural motifs by members of the PDGF/VEGF family of growth factors allows the proteins to exert their growth promoting or mitogenic effects. Aaronson Declaration I at ¶3. Applicants direct the Examiner's attention to Hannick et al., 1986, Molecular and Cellular Biology 6(4):1304-1314 ("Hannick"), a publication discussed in Aaronson Declaration I (previously submitted in the instant application). As demonstrated by Aaronson Declaration I, Hannick would provide to one skilled in the art knowledge that retention of the structural motif conserved among members of the PDGF/VEGF family (i.e., the region spanning eight strictly conserved cysteine residues) is critical for maintaining biological activity in PDGF. Aaronson Declaration I at ¶¶7, 8. Hannick demonstrates a consistent correlation between the retention of the biological properties of PDGF

and the presence of the structural motif of eight strictly conserved cysteine residues shared among the members of the PDGF/VEGF family. Hannick at page 1304, col. 2, 1. A series of C-terminal deletion mutants were generated and analyzed for their ability to retain biological activity. The results demonstrated that the region spanning the eight cysteine residues strictly conserved among the members of the PDGF/VEGF family defined a region of PDGF critical to retain PDGF biological activity, i.e., 103 amino acids extending from residue 112 to residue 214 of the B chain of PDGF (as shown in Figure 6 of Hannick). *See* Hannick at page 1311, col. 2.

Furthermore, as discussed in Aaronson Declaration I, post-filing references corroborate the teachings of the instant specification. Alitalo et al., U.S. Patent No. 6,361,946 provides experimental results demonstrating that recombinant polypeptides having at least the amino acid residues of VEGF-2 spanning the eight strictly conserved cysteine residues, shared by all known members of the PDGF/VEGF family, retain biological activity. Such biological activities include the ability to autophosphorylate VEGF-2 receptors, stimulate proliferation and migration of endothelial cells, and increase permeability of blood vessels. Accordingly, such results corroborate the expectation of one skilled the art that fragments having at least amino acid residues 108-188 of VEGF-2 (as shown in SEQ ID NO:2) would retain biological activity. *See* Aaronson Declaration I at ¶¶7, 12, and 15.

In summary, the instant specification enables the claimed invention. The teachings of the specification allow one skilled in the art to recognize that the retention of the structural motif of eight conserved cysteine residues in VEGF-2 would be important to retain biological activity. Given the teachings of the specification coupled with the knowledge that retention of the conserved cysteine motif in other members of the PDGF/VEGF family is important for retention of biological activity, (e.g., PDGF), one skilled in the art as of the earliest priority date of the instant application would reasonably expect that the retention of the same structural motif in VEGF-2, a new member of the PDGF/VEGF family, would also be important for biological activity. Accordingly, one skilled in the art would recognize and expect that amino acid residues 108 to 188 of SEQ ID NO:2 would be important for retention of biological activity, including stimulation of proliferation of endothelial cells and angiogenesis.

Under the applicable legal standard for enablement, one skilled in the art could therefore make and use the claimed invention, without undue experimentation, based on the disclosure in the patent specification coupled with information known in the art at the time the patent application was filed. *U.S. v. Telectronics Inc.*, 857 F.2d 778, 8 U.S.P.Q.2d 1217 (Fed. Cir.

1988). In view of the foregoing, Applicants submit that the instant specification enables the polypeptides of claims 146 and 165 that possess biological activity and would therefore be useful in the methods of stimulating endothelial cell proliferation and angiogenesis as claimed. Claims 146-156 and 166-176 depend from claims 145 and 165, respectively, and are thus enabled by virtue of their dependency. Applicants request that the Examiner reconsider and withdraw the rejection of the instant claims under 37 U.S.C. § 112, first paragraph.

ii. Fragments with Activity

Applicants respectfully submit that claims 225-236, 245-256, 265-276, 285-296, 407-418 427-438, which are directed towards fragments of SEQ ID NO:2, ATCC Deposit No. 97149, or ATCC Deposit No. 75698 having a specified activity (either angiogenic activity or endothelial cell proliferative activity) are enabled by the specification.

The Examiner rejected these claims on the grounds that the "function of these protein fragments" is not taught in the specification and "it is not predictable to one of ordinary skill in the art what residues can be altered while still providing the desired peptide function" (Paper No. 28, page 7). Specifically, the Examiner asserts that:

> proteins which are smaller than the full-length protein of the invention would have one or more amino acid substitutions, deletions, insertions and/or addition... Applicants have provided no guidance or working examples of the functions of these protein fragments [are], or what amino acids can be altered (added, substituted, or deleted) in order to maintain the desired activity of these fragments. Furthermore, it is not predictable to one of ordinary skill in the art what residues can be altered while still providing the desired peptide function.

(Paper No. 28, page 7). Applicants respectfully traverse.

a. The Specification Teaches the "Function" of the Claimed Fragments

Contrary to the Examiner's assertion, many functions of the claimed fragments are described in the specification, including the use of "VEGF-2, or biologically active portions thereof" to promote angiogenesis and endothelialization (*See,* specification, page 27, line 20 through page 28, line 24). Furthermore, independent claims 225, 245, 265, 285, 407 and 427 specifically recite the activity of the claimed fragments, either endothelial cell proliferative activity or angiogenic activity.

b. The Specification Provides Guidance for Amino Acid Substitutions

The specification provides guidance to the skilled practitioner as to what amino acids can be altered while maintaining the desired activity. For example, Table 1, found on page 20 of the specification, teaches conserved amino acid substitutions. AT page 18, lines 5-6, the specification describes "substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids." Also, on page 18, lines 16-17, the specification references Bowie, et al., which teaches phenotypically silent amino acid, changes. On page 18, line 19 to page 19, line 2, the specification teaches that VEGF-2 fragments, derivatives, or analogs may be:

> (i) one in which one or more of the amino acid residues are substituted with a conserved o r n onconserved a mino a cid r esidue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code; or (ii) one in which one or more of the amino acid residues includes a substituent group; or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol); or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence; or (v) one in which comprises fewer amino acid residues shown in SEQ ID NOS:2 or 4, and retains the conserved motif and yet still retains activity characteristics of the VEGF family of polypeptides. \

Additionally, Applicants submit that at the time the instant specification was filed, it was common in the art to make changes to proteins through substitutions, deletions, insertions and/or additions. This is supported by references in the specification to publications such as Bowie et al. (Specification, page 18, lines 16-17) and Ostade et al. (Specification, page 19, line 15), which demonstrate the skill in the art at the time the application was filed.

c. One Skilled In the Art Would Reasonably Expect that Amino Acid Residues 108-188 would be Important for Biological Activity

As discussed above, the specification teaches VEGF2 is a new member of the PDGF/VEFG family that includes a conserved structural motif of eight cysteine residues, spanning amino acids 108-188 (*See,* SEQ ID NO:2 and FIG.3), which is important for biological activity. In addition to the conserved eight cysteine residues, a signature consensus sequence for

the PDGF/VEGF family, PXCVXXXRCXGCCN, which corresponds to amino acid residues 131 to 144 of VEGF-2 (as shown in SEQ ID NO:2), is also conserved in VEGF-2 (specification at page 9, lines 12-14). Therefore, based on the teachings of the specification, one skilled in the art would recognize the significance of retaining the structural motif spanning amino acid residues 108-188 for maintaining biological activity.

Furthermore, Applicants have provided a Declaration of Dr. Stuart Aaronson ("Aaronson Declaration I"), which demonstrates that, at the time the application was filed, it was known by those of skill in the art that the retention of conserved structural motifs by members of the PDGF/VEGF family of growth factors allows the proteins to exert their growth promoting or mitogenic effects. Aaronson Declaration I at ¶3.

Furthermore, as discussed above, scientific publications by others (*i.e.,* Hannick et al. and Alitalo et al) demonstrate that retention of the structural motif conserved among members of the PDGF/VEGF family is important and corroborate the teachings of the instant specification by providing experimental results demonstrating that polypeptides having at least the amino acid residues of VEGF-2 spanning the eight strictly conserved cysteine residues, shared by all known members of the PDGF/VEGF family, retain biological activity.

Therefore, based on the teachings of the specification, one would understand that VEGF-2 is a member of the PDGF/VEGF family of growth factors and would therefore recognize that the retention of the structural motif would be important for maintaining biological activity. Although Applicants maintain that the claims are enabled as pending, in the interest of furthering prosecution, claims 225, 245, 26, 285, 407 and 427 have been amended to recite "wherein said protein fragment comprises amino acid residues SEQ ID NO:8."

iii. Fragments of at least 30 Contiguous Amino Acids

Applicants respectfully submit that claims 305-317 and 326-338, which are directed towards protein fragments of at least 30 contiguous amino acids of SEQ ID NO:2 or ATCC Deposit No. 97149, are enabled by the specification.

At page 21, lines 13-14, the specification discloses fragments "containing at least 30 amino acids and more preferably at least 50 amino acids" of VEGF-2. From the disclosure, Applicants respectfully submit that one of skill in the art would be able to make fragments containing at least 30 or 50 amino acids of SEQ ID NO:2.

Applicants also contend the disclosure provides sufficient guidance such that one of skill in the art would be able to use the claimed fragments. Applicants respectfully point out that it is not necessary for the claimed polypeptides to be "biologically active" or to be defined by "functional properties," to be used. In fact, the polypeptides recited in independent claims 305 and 326, have several uses asserted in the specification that do not require biological activity. For example, the specification discloses that fragments containing 30 contiguous residues of SEQ ID NO:2 or ATCC Deposit No. 97149 may be used as "intermediates" for producing the "corresponding full-length polypeptide by peptide synthesis" (Specification, page 21, lines 18-23). The specification also teaches that fragments can be used to generate antibodies. For example, at page 40, lines 27-29, the specification teaches that fragments "can be used as an immunogen to produce antibodies thereto." At page 41, lines 7-9, the specification teaches that "even a sequence encoding only a fragment of the polypeptide can be used to generate antibodies binding the whole native polypeptide." Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide. The specification also describes methods for generating such antibodies. *See,* specification, page 41, lines 4-10 (antibodies "can be obtained by direct injection of the polypeptide into an animal").

Although Applicants maintain that the specification teaches the skilled artisan how to make and use the claimed fragments as pending, in the interest of furthering prosecution, Applicants have canceled claims 305-317 and 326-338, along with the corresponding method claims (*i.e.,* claims 318-325 and 339-346, respectively) without prejudice or disclaimer. Applicants therefore request withdrawal of this rejection.

5. Written Description Rejections Under 35 U.S.C. §112, First Paragraph

A. Fragments

The Examiner rejected claims 133-156, 165-176, 225-236, 245-256, 265-276, 285-296, 305-317, 326-338, 407-418, and 427-438 under 35 U.S.C. §112, first paragraph for lacking written description. Specifically, the Examiner asserts that the claims are genus claims and the specification and claims do not indicate what distinguishing attributes are shared by the members of the genus. Applicants respectfully disagree and traverse.

Applicants submit that the instant specification describes an adequate number of species to support the genus claims and describes the claimed invention in a manner that shows that the inventors had possession of the invention at the time the instant application was filed.

i. Specific Fragments

As discussed above, claims 133-156 and 165-176 are directed towards specific fragments of SEQ ID NO:2 (*i.e.*, amino acids 131 to 144, 71 to 396, or 24 to 396).

A patent specification, in order to satisfy the written description requirement, must describe the claimed invention in sufficient detail to allow a skilled artisan to reasonably conclude that the inventor had possession of the claimed invention at the time the application was filed. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563 (Fed. Cir. 1991).

Applicants respectfully submit that the specification describes the invention of claim 133 in sufficient detail to allow a skilled artisan to reasonably conclude that Applicants had possession of the claimed invention at the time the application was filed. The specification discloses that "[i]t is particularly important that all eight cysteines are conserved within all four members of the [VEGF/PDGF] family (see boxed areas of FIG. 3)" (*See,* specification, page 9, lines 11-12). One "boxed area" of FIG. 3 corresponds to amino acid residues 131-144 of SEQ ID NO:2, recited in claim 133. Thus, one of skill in the art would clearly recognize that Applicants had possession of the invention recited in claim 133 at the time the application was filed. Although Applicants maintain that the invention of claim 133 is supported by sufficient written description in the specification, in the interest of advancing prosecution on the merits, applicants have canceled claims 133-144 without prejudice or disclaimer.

Similarly, the specification provides sufficient written description of the subject matter of claim 145. The specification discloses that SEQ ID NO:4 is "a truncated, biologically active form of VEGF2," which "comprises approximately 350 amino acid residues of which approximately the first 24 amino acids represent the leader sequence" (*See,* specification, page 6, lines 8-11). When SEQ ID NO:4 is compared to SEQ ID NO:2, it is apparent that SEQ ID NO:4 corresponds to the C-terminal 350 amino acids of SEQ ID NO:2. Without the 24 amino acid leader sequence, SEQ ID NO:4 corresponds to amino acid residues 71 to 396 of SEQ ID NO:2, recited in claim 145. Therefore, Applicants respectfully submit that one of skill in the art would be able to reasonably conclude that Applicants had possession of the invention recited in claim 145 at the time the application was filed.

Written description for claim 165 is provided in the description of FIGS. 12 and 13, found on page 27, lines 13-15. The specification discloses that a "VEGF-2 polypeptide of SEQ ID NO:2, minus the initial 46 amino acids, is a potent mitogen for vascular endothelial cells and stimulates their growth and proliferation." A "VEGF-2 polypeptide of SEQ ID NO:2, minus the initial 46 amino acids" corresponds to a fragment comprising amino acids 24 to 396 of SEQ ID NO:2, recited in claim 165. Consequently, one of skill in the art would be able to reasonably conclude that Applicants had possession of the invention recited in claim 165 at the time the application was filed.

Because the specification describes the invention of claims 133, 145 and 165 "in sufficient detail to allow a skilled artesian to reasonably conclude that the inventor had possession of the claimed invention at the time the application was filed, a rejection of claims 133, 145 and 165, under 35 U.S.C. § 112, first paragraph is not proper. Claims 134-144, 146-156 and 166-176 depend from claims 133, 145 and 165, respectively, and are supported by adequate written description by virtue of their dependency. Applicants request withdrawal of this rejection.

ii. Fragments with Activity

Claims 225-236, 245-256, 265-276, 285-296, 407-418 427-438 are directed towards fragments having a specified activity, either angiogenic activity or endothelial cell proliferative activity.

The Examiner has rejected these claims on the grounds that "[s]tructural features that could distinguish compounds in the genus from others in the nucleic acid or protein class are missing." Applicants respectfully disagree and traverse.

The specification describes structural features important in maintaining VEGF-2 biological activity. In particular, as discussed above, the specification specifically describes the conserved functional motif of eight cysteine residues and the consensus sequence PXCVXXXRCXGCCN. Applicants submit that this disclosure provides a description of a specific structural feature that can be used to distinguish members of the genus and that one of skill in the art would recognize that Applicants were in possession of the common structural feature at the time the application was filed.

Although Applicants maintain that the claims are supported by an adequate written description as pending, in the interest of furthering prosecution, claims 225, 245, 265, 285, 407

and 427 have been amended to recite "wherein said protein fragment comprises SEQ ID NO:8." Applicants therefore request withdrawal of this rejection.

iii. At least 30 Contiguous Amino Acids

Claims 305-317 and 326-338 are directed towards protein fragments of at least 30 contiguous amino acids of SEQ ID NO:2 or the cDNA contained in ATCC Deposit No. 97149.

The Examiner contends that the claims are not supported by a written description because "the skilled artisan cannot envision all the contemplated nucleic acid sequence possibilities" Applicants respectfully disagree.

The present application discloses fragments "containing at least 30 amino acids and more preferably at least 50 amino acids" of VEGF-2 (Specification, page 21, lines 13-14). Applicants contend that the skilled artisan could clearly envision each polypeptide comprising at least 30 contiguous amino acids of SEQ ID NO:2 as a progression, *i.e.*, polypeptides comprising nucleotides 1-30, 2-31, 3-32, etc. The skilled artisan could certainly further envision sequentially adding contiguous amino acids to either end of any of the described embodiments. Indeed, nothing more than what is described in the specification would be required for the skilled artisan to identify every single one of the polypeptides and polypeptide fragments containing at least 30 amino acids of SEQ ID NO:2. Thus, it would be readily apparent to the skilled artisan that the Applicants had "invented what is claimed." *Vas-Cath, Inc v. Mahurkar*, 935 F.2d 1555, 1563 (Fed. Cir. 1991).

Furthermore, the Federal Circuit court has recently indicated that the written description requirement for generic claims to genetic material, such as cDNA, may be satisfied by providing a recitation of structural features common to a substantial portion of the members of the genus. *University of California v. Eli Lilly*, 119 F.3d 1559, 1569 (Fed. Cir. 1997). It logically follows that claims to polypeptides may also be satisfied by providing a recitation of structural features common to a substantial portion of the members of the genus. Applicants maintain that the description of the reference polypeptide SEQ ID NO:2 provides one skilled in the art with the necessary recitation of a structural feature common to members of the genus. Applicants respectfully submit that the recitation of the reference protein is a recitation of a structural feature common to the members of the claimed genus, *i.e.*, the proteins included within the claimed genus will each have at least 30 contiguous amino acids in common to the reference polypeptide. As discussed above, once one of ordinary skill in the art is enlightened by the

specification and provided with the reference polypeptide sequence, the skilled artisan could readily envision and derive any number of polypeptides consisting of at least 30 contiguous amino acids of the reference polypeptide sequence. Therefore, the specification clearly conveys that Applicants were in possession of the claimed invention on the priority date of the instant application.

In view of the comments above, Applicants respectfully submit that claims 305-317 and 326-338 are supported by an adequate written description as pending. However, in the interest of furthering prosecution, Applicants have canceled claims 305-317 and 326-338, along with the corresponding method claims (*i.e.*, claims 318-325 and 339-346, respectively) without prejudice or disclaimer. Applicants therefore respectfully request that the rejection be reconsidered and withdrawn.

B. Mature or Proprotein

The Examiner further rejected claims 33-44, 53-64, 73-84, 93-104, 347-358, and 367-378 for lack of written description because "the instant specification fails to describe that portion of a protein which is the 'mature' portion, or what constitutes a 'proprotein'." Paper No. 28, page 9. Applicants respectfully disagree and traverse.

In the specification, "proprotein" and "mature" portions of VEGF-2 are specifically described as follows:

> The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Specification, page 11, lines 11-21. Additional description of the "mature" and "proprotein" forms can be found at page 9, lines 5-8 and page 10, lines 4-17.

From the teachings in the specification, one skilled in the art could readily envision and identify polypeptides that fall within the recited "mature" and "proprotein" terminology. Applicants therefore submit that the specification provides adequate written description for the terms "mature" and "proprotein" sequences.

Furthermore, the M.P.E.P. states that by "disclosing in a patent application a device that *inherently* performs a function or has a property, operates according to a theory or has an advantage, a *patent application necessarily discloses* that function, theory or advantage, even though it says nothing explicit concerning it." M.P.E.P. § 2163.07(a).

Applicants submit that the mature portion of the protein is inherently disclosed in the patent application through its teachings. All of the information required for one of skill in the art to obtain the mature processed form of a polypeptide is found within the amino acid sequence of the precursor form of the polypeptide. The nucleotide sequence of the precursor form of VEGF-2 inherently contains all of the motifs required for the cell to process the protein to a mature form of VEGF-2. As demonstrated by the Declaration of Dr. Stuart Aaronson ("Aaronson Declaration II"), which was submitted in co-pending U.S. Patent Application Serial No. 08/107,997 and is appended hereto as **Attachment D**, the capacity of the VEGF-2 polypeptide to be expressed and proteolytically processed to the mature form of the protein is a natural and intrinsic property of that molecule. The expression and proteolytic processing of the VEGF-2 polypeptide to the mature form of the protein is a result of the cell's recognition of the "signals" present in the amino acid sequence of the precursor form of the polypeptide. Thus, one of skill in the art, armed with the teaching of the specification would have all the information required to express and isolate a mature and biologically active form of VEGF-2.

The Examiner also contends that "the structure of a 'mature form of a polypeptide' cannot be predicted on the basis of the amino acid sequence of the entire protein since the protein may be proteolytically cleaved in vivo, as well as being differentially processed based on which in [sic] tissue the protein is expressed." (Paper No. 25, page 8). However, the Examiner fails to appreciate that the mature, processed form is an inherent property of the precursor form of VEGF-2, and its capacity to be processed is also an inherent, intrinsic property which does not vary. As demonstrated by Aaronson Declaration II, it is not unexpected that the processing of the precursor form may vary depending on the expression system used (See ¶12, of Aaronson Declaration II). However, it is unnecessary for the patent specification to specifically describe either the mechanics of processing or each of the resulting processed forms, as the processing is

an automatic and natural event. Thus, other than provide the amino acid sequence of the precursor form, no other information is required for one of skill in the art to achieve the mature form, nor is more information required for one to recognize that the applicants were in possession of the claimed invention.

The function of the "written description" requirement of 35 U.S.C. 112, first paragraph, is to ensure that applicants had possession of the claimed subject matter, as of the filing date of application relied on. *In re Blaser*, 556 F.2d 534, 194 USPQ 122 (CCPA 1977). The inquiry into satisfaction of the written description requirement is factual and depends on the nature of the invention and the amount of knowledge imparted to those of skill in the art by the disclosure. *In re Wertheim*, 646 F.2d 527, 191 USPQ 90 (CCPA 1976). Satisfaction of the "written description" requirement does not require *in haec verba* antecedence in the originally filed application. *In re Lukach*, 440 F.2d 1263, 169 USPQ 795 (CCPA 1971). The written description requirement can be satisfied by showing that the disclosed subject matter, when given its 'necessary and only reasonable construction,' inherently (*i.e.*, necessarily) satisfies the limitation in question. *Staehelin v. Secher,* 24 USPQ2d, 1513, 1520 (Bd. Pat. Int'f. 1992) ("a specification need not describe the exact details for preparing every species within the genus described"). In general, precedent establishes that although the applicant 'does not have to describe exactly the subject matter claimed, the description must clearly allow persons of skill in the art to recognize that [the applicant] invented what is claimed.' *In re Gosteli,* 872 F.2d 1008,1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989).

Applicants invite the Examiner's attention to Aaronson Declaration II, submitted herewith as **Attachment D**, as evidence that one of ordinary skill in the art would recognize that the applicants were in possession of the claimed invention as of the March 8, 1994 filing date of the priority application serial no. 08/207,550 (*In re Alton*, 76 F.3d 1168 (Fed. Cir. 1996)). As shown by Aaronson Declaration II, the precursor form of a polypeptide intrinsically contains the signals required to result in the processing to the mature form of the protein (See Aaronson Declaration II at ¶¶13 to 17). As evidenced by ¶¶6 to 12 of Aaronson Declaration II, the 350 amino acid sequence of VEGF-2 contains all of the information and motifs required to allow the host cell to process the 350 amino acid sequence to the mature form of VEGF-2. The molecular weight of the mature form of VEGF-2 expressed and obtained by the cell is an inherent property that is the result of proteolytic processing of the precursor form of VEGF-2 (See Aaronson Declaration II at ¶¶13 to 17). Indeed, as provided by Aaronson Declaration II, both the 350

amino acid form and the 419 amino acid form of VEGF-2 are identically processed to the mature form of VEGF-2, resulting in the secretion of polypeptides of identical molecular weights, as assessed by SDS PAGE.

To overcome a prima facie case of unpatentability under 35 U.S.C. §112, first paragraph, the applicants must show by evidence or argument that the invention as claimed is adequately described to one of ordinary skill in the art. *In re Alton* 76 F.3d 1168, 1175 (Fed. Cir. 1996). Aaronson Declaration II provides evidence that one of skill in the art would recognize that the mature form of VEGF-2 is adequately described by the instant application. If a person of ordinary skill in the art would have understood the inventor to have been in possession of the claimed invention at the time of filing, even if every nuance of the claim is not explicitly described in the specification, then the adequate written description requirement is met. *In re Alton* 76 F.3d 1168, 1175 (Fed. Cir. 1996). The Federal Circuit has noted that the priority application need not use the identical words to describe the claimed invention, if it shows the subject matter claimed with an adequate direction as to how to obtain it. *Kennecott v. Kyoura International, Inc.* 835 F.2d 1419. 1422, 5 USPQ 2d 1194, 1197 (Fed. Cir. 1987), *cert denied*, 486 U.S. 1008 (1988).

In this instance, the priority application and the instant application clearly describe the subject matter of the invention and also provide adequate direction as to how to obtain the mature form of VEGF-2. Furthermore, as evidenced by Aaronson Declaration II, one of ordinary skill in the art would recognize that the applicants were in possession of the claimed invention. Indeed, it is unnecessary for the specification to explicitly define by amino acid sequence, the beginning and end of the processed, mature form of VEGF-2 in order for one skilled in the art to recognize a "mature portion of a protein." The specification teaches the "mature portion" of VEGF-2 because the "mature portion" of VEGF-2 is naturally and inherently produced when expressed by a host cell.

Thus, the instant specification, and the specification of the priority application, contains sufficient information required of one of ordinary skill in the art to recognize that the applicants were in possession of the invention as claimed. Hence, the rejection under 35 U.S.C. §112, first paragraph, should be withdrawn.

CONCLUSION

In view of the foregoing remarks, Applicants believe they have fully addressed the Examiner's concerns and that this application is now in condition for allowance. An early notice to that effect is urged. A request is made to the Examiner to call the undersigned at the phone number provided below if any further action by Applicants would expedite allowance of this application.

If there are any fees due in connection with the filing of this paper, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under 37 C.F.R. §1.136 not accounted for above, such an extension is requested and the fee should be charged to our Deposit Account.

Dated: February 3, 2003

Respectfully submitted,

By _____
Melissa Jean Pytel (Reg. No. 41,512)
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Enclosures
MMW/MJP/AKR/lcc

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of: Hu et al.

Application No.: 09/219,442-Conf. #4797 Group Art Unit: 1647

Filed: December 23, 1998 Examiner: R. Landsman

For: Vascular Endothelial Growth Factor 2

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the claims:

Please show the amendments to the claims as follows:

225. (Amended) A purified protein fragment produced by the method comprising:
(a) expressing a protein fragment of SEQ ID NO:2 from a host cell, wherein said protein fragment comprises SEQ ID NO:8 and has angiogenic activity; and
(b) recovering said protein fragment.

245. (Amended) A purified protein fragment produced by the method comprising:
(a) expressing a protein fragment encoded by the cDNA contained in ATCC Deposit No. 97149 from a host cell, wherein said protein fragment comprises SEQ ID NO:8 and has angiogenic activity; and
(b) recovering said protein fragment.

265. (Amended) A purified protein fragment produced by the method comprising:
(a) expressing a protein fragment of SEQ ID NO:2 from a host cell, wherein said protein fragment comprises SEQ ID NO:8 and has endothelial cell proliferative activity; and
(b) recovering said protein fragment.

285. (Amended) A purified protein fragment produced by the method comprising:

(a) expressing a protein fragment encoded by the cDNA contained in ATCC Deposit No. 97149 from a host cell, wherein said protein fragment comprises SEQ ID NO:8 and has endothelial cell proliferative activity; and

(b) recovering said protein fragment.

407. (Amended) A purified protein fragment produced by the method comprising:

(a) expressing a protein fragment encoded by the cDNA contained in ATCC Deposit No. 75698 from a host cell, wherein said protein fragment comprises SEQ ID NO:8 and has angiogenic activity; and

(b) recovering said protein fragment.

427. (Amended) A purified protein fragment produced by the method comprising:

(a) expressing a protein fragment encoded by the cDNA contained in ATCC Deposit No. 75698 from a host cell, wherein said protein fragment comprises SEQ ID NO:8 and has endothelial cell proliferative activity; and

(b) recovering said protein fragment.

AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent Application Serial No. 696764 by Human Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by Ludwig Institute for Cancer Research

STATUTORY DECLARATION

I, Kari Alitalo, a Research Professor of the Finnish Academy of Sciences, at The Molecular/Cancer Biology Laboratory, Biomedicum Helsinki, P.O.B. 63 (Haartmaninkatu 8) 00014 University of Helsinki, Finland, do solemnly and sincerely declare as follows:

I. INTRODUCTION

1.1 In July, 2002, I executed a third statutory declaration (hereinafter referred to as "OKA3" (Opponents, Kari Alitalo, 3rd Declaration)) to provide experimental evidence in support of the opposition filed by Ludwig Institute for Cancer Research ("Ludwig Institute") to the issuance of a patent to Human Genome Sciences, Inc., ("HGS") based on HGS's Australian Patent Application No. 696764 ("the opposed application"). A brief summary of the various declarations that have been filed throughout these proceedings was provided in OKA3 and will not be repeated here.

1.2 I hereby reaffirm my understanding that I have an overriding duty to the Patent Office (and to any Australian Federal Court that should review the Patent Office decision) to provide objective scientific analysis that I believe to be truthful. I hereby affirm that, to the

best of my knowledge and belief, factual statements herein are true and opinion statements herein represent my objective scientific opinion and analysis.

1.3 As described in OKA3, the purpose of my third statutory declaration was to design and perform protein expressi n experiments that would address any criticisms raised in the second series of declarations filed on behalf of HGS, particularly the second declarations of Dr. Nicholas Hayward (ANH2) and Dr. Stuart Aaronson (ASA2). Further, Ludwig Institute asked me to comment on the data from expression studies performed by Dr. Susan Power and reported in her second declaration (hereinafter "ASP2"), filed at the same time as ANH2 and ASA2.

1.4 The purpose of this declaration, hereinafter referred to as "OKA4", is to supplement the findings in OKA3 with an additional figure that depicts the results of transfection experiments for the Australian Patent Office. The results described in this declaration further demonstrate that the negative results are due to expression and secretion defects in VEGF-2 (as taught in the opposed application) rather than variations in transfection efficiency (as suggested, wrongly, by various HGS declarants). Also, I have attempted to further articulate the benefit of using two different transfection-efficiency controls (beta-galactosidase and luciferase) in OKA3 and again here in OKA4.

1.5 Although the experimental design reported in OKA3 and the experimental design of the present declaration are very similar, the protocol described below included an extra adjustment in immunoprecipitation sample sizes, to normalize for apparent modest experimental variation in transfection efficiency or other factors influencing the quantity of recombinant protein expression over time. (See paragraphs 4.1 - 5.5, below.) Other minor changes were made in the experimental protocol of the present declaration (e.g., times of incubations) due to time constraints and do not affect the results of the types of experiments performed and described herein. The experiments described herein provide further evidence that cells cannot express and secrete VEGF-2 as taught in the opposed application. On this point, the results were the same as the data already presented in OKA1, OKA2, and OKA3, further confirming that these declarations were accurate and were experimentally sound.

II. EXPERIMENTAL PROCEDURE

A. Cells and Plasmids

2.1 Results reported in OKA2 revealed that COS and 293T cells were equally appropriate cell lines for analyzing VEGF-2 protein expression and secretion. For these experiments, 293T cells were grown in DMEM supplemented with 10 % fetal bovine serum, glutamine and penicillin/streptomycin.

2.2 The polymerase chain reaction (PCR) was employed to construct a cDNA fragment that corresponded to amino acids 70 to 419 of prepro-VEGF-C. For the purpose of these experiments (directed to assessing transfection efficiency and protein expression at various time points) the cDNA fragment encoding amino acid residues 70 to 419 of prepro-VEGF-C corresponds appropriately with the cDNA encoding the full length sequence of the VEGF-2 polypeptide described in the opposed application. Nucleotides 559 to 1608 of the VEGF-C cDNA (Reported in Document D70, Joukov et. al. 1996, GenBank accession number X94216) were PCR amplified with the primers 5'-CGCGGATCCATGACTGTACTCTACCCA-3' containing a BamHI site and 5'-CGCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGG-GTACTCGAGGCTCATTTGTGGTCT-3' containing a XhoI site, HA-tag, a stop codon and a XbaI site and cloned into pcDNA1(Amp)-vector (Invitrogen). The resultant vector was designated as VEGF-2(HGS)/pcDNA1.

2.3 An expression vector was also constructed that contained the full length (419 codons) VEGF-C sequence (OKA2 at 3.3.3) for use as a positive control in the expression and secretion analyses. The resultant vector was designated as VEGF-C/pcDNA1.

B. Transfection and time course:

2.4 A principle criticism alleged by the HGS experts was that my OKA2 declaration failed to include transfection efficiency data (ANH2 at 1.5; ASA2 at 24). Thus, for these new experiments, two separate expression vectors, pRL expressing Renilla Luciferase (Promega) and pCMV-x-gal expressing beta-galactosidase under CMV promoter, were used as transfection controls.

2.5 The other principle criticism of the procedures reported in my OKA2 declaration was regarding the lack of time points in the expression analyses (ANH2 at 1.5; ASA2

at 25). To address this concern, three different time points were tested in the new experiments. In particular, the 293T cells were split 1:6 and fresh medium was changed 19 hours thereafter. Three hours after medium change, VEGF-2(HGS)/pcDNA1, VEGF-C/pcDNA1, or empty vector were co-transfected with either pRL (three plates with each combination in a 16:1 ratio), or pCMV-x-gal (one plate with each combination in a 1:1 ratio), using FuGENE6 Transfection Reagent (Roche). The conditioned media and the cells were harvested 24 hours, 48 hours, or 74 hours after the transfection for the purpose of evaluating protein expression and secretion at these different time points. Either twenty-four hours (for time points 48h and 74h) or eight hours (for time point 24h) prior to harvesting, the cells were washed twice with PBS and changed to 3 ml of MEM medium containing 100 µCi/ml ^{35}S-methionine and ^{35}S-cysteine (Promix, Amersham) for metabolic labeling of proteins synthesized by the cells[1]. At the indicated time points the conditioned media was harvested and cleared by centrifugation. The cells were trypsinized, washed twice with PBS and lysed in 1x passive lysis buffer from the Dual-Luciferase Reporter Assay System (Promega).

C. **Beta-galactosidase staining:**

2.6 The cells were washed twice with PBS, fixed with 0.05% glutaraldehyde in PBS for 15 minutes at room temperature, washed three times with PBS, and stained over night with 2.5 mg/ml x-Gal (5-bromo-4-cloro-3-indolyl-ß-D-galactopyranoside) at 37°C. The beta galactosidase data provided evidence of transfection efficiency.

D. **Luciferase assay:**

2.7 The protein concentrations of the cell lysates were determined by using the BCA Protein Assay (Pierce). To quantify expression of recombinant protein in transfected cells, luciferase activity in cell lysates was measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase data provided evidence of transfection efficiency and recombinant protein expression efficiency at various time points.

[1] See explanation in OKA2 at 3.4.2

E. Immunoprecipitation:

2.8 Immunoprecipitation experiments were conducted to identify the presence of VEGF-C or VEGF-2 polypeptides in the conditioned media from the cells after the indicated incubation times.

2.9 For immunoprecipitation, aliquots of each conditioned medium were supplemented with BSA and Tween 20 to final concentrations of 0.5% and 0.02%, respectively. The different VEGF-2 or VEGF-C peptides were immunoprecipitated with polyclonal antibodies raised against a synthetic peptide corresponding to amino acid residues 104-120 of the VEGF-C prepropeptide (Antisera 882, reported in Document D71, Joukov et al., 1997) at 4 °C overnight. This peptide is present in the secreted form of VEGF-C, and the opposed application teaches that it should be present in mature VEGF-2 as well. Thus, antisera raised against this peptide should recognize VEGF-2 or VEGF-C polypeptides produced by the cells.

2.10 The immunocomplexes of secreted polypeptides bound to antisera 882 were precipitated with protein A-Sepharose for 2 hours and washed 2 times with 1X binding buffer (0.5% BSA, 0.02% Tween20 in PBS) and once with PBS at 4°C. The proteins were analyzed by SDS-PAGE in a 12 % gel under reducing conditions.

III. EXPERIMENTAL RESULTS AND ANALYSIS

A. Beta-galactosidase and transfection efficiency

3.1 In order to analyze transfection efficiency, the constructs of interest (VEGF-2(HGS)/pcDNA1, VEGF-C/pcDNA1, or the mock vector) were co-transfected into 293T cells with a plasmid encoding beta-galactosidase. Beta-galactosidase is an enzyme that causes production of a colorometric product, under the assay conditions used. In particular, successful transfection with the beta-galactosidase plasmid is observable as a colored colony on a culture plate. The presence and percentage of colored cells that are observable following the transfection experiments provides a measurement of relative transfection efficiency for the different transfections. HGS's declarant, Dr. Power, chose a beta galactosidase for her transfection efficiency control in her ASP2 declaration (See ASP2 at 20 and 24).

3.2 Ludwig Institute asked that I provide actual data in this declaration for the transfection efficiency study. Accordingly, I have included photographic results of the beta-

galactosidase staining as Figure 1B attached hereto (see Exhibit KA-1 hereto). The figure represents photographs of culture plates following plating, transfection, and growth of the cells. Dark colored "spots" represent colonies formed from cells that were successfully transfected with the beta galactosidase vector and that are therefore expressing beta-galactosidase and appear dark blue under the assays conditions used. White or colorless cells, on the other hand, are not expressing the beta-galactosidase, and are scored as negative with respect to transfection.

3.3. The photographs in Figure 1B reveal that each of the transfections were very successful (numerous dark colored colonies were observed on all of the plates), and that the percentage of transfected cells were comparable in each of the three transfection groups (i.e., VEGF-2, VEGF-C, and mock). This data is sufficient to conclude that transfection efficiency is not confounding the results of my comparative expression studies, thus dispelling one of HGS's main criticisms of my OKA2 declaration.

B. Luciferase and Protein expression

4.1 The luciferase experiments provide a supplemental control for transfection efficiency that complements the beta-galactosidase study, and also provide an indication of the level of recombinant protein expression in transfected cells. The luciferase study was comparable to the beta-galactosidase study in the sense that it involved co-transfection of the constructs of interest (VEGF-2 or VEGF-C plasmid) with a second construct, the presence of which was measurable by a visualization assay. Because the co-transfected luciferase plasmid encodes an enzyme that causes production of light waves under the assay conditions that were used, the measurements of light units provides an indication that the transfections were successful. However, the luciferase assay differs from the beta-galactosidase assay because it quantitatively measures *recombinant protein* from transfection plates, whereas the beta galactosidase assay only measures the number of transfected versus non-transfected colonies, and does not provide a quantitative measurement of the amount of recombinant protein that the cells are producing.

4.2 The VEGF-2(HGS)/pcDNA1, VEGF-C/pcDNA1, or the mock vector were co-transfected into 293T cells with the plasmid encoding the Renilla luciferase gene as described above. At the datapoints selected for the experiment (24, 48, and 74 hours after transfection) conditioned medium was collected to assay for the secretion of VEGF-2 or VEGF-

C by the cells, and cell lysates were also analyzed to quantify the luciferase activity in the cells at the same time points.

4.3 The results of the luciferase assay are presented as a bar graph in Figure 1A (see Exhibit KA-1 hereto), showing relative light units per microgram of protein for each of the three transfection groups at each of the time points specified. Since the luciferase assay is a measure of recombinant protein expression, it provides a second indication that both the VEGF-2 and the VEGF-C transfections were successful. because all groups showed luciferase activity at all time points. The fact that the relative levels of luciferase (in the VEGF-2 versus VEGF-C cultures) varies somewhat with time reflects the fact that the luciferase measurements are quantitative for the amount of recombinant, active luciferase protein present, rather than quantitative for percentage of cells successfully transfected. At all time points studied, the luciferase measurements in the VEGF-2 and VEGF-C plates were of the same order of magnitude.

C. **Analysis of VEGF-2 and VEGF-C expression and secretion.**

5.1 At the outset, it is my opinion that the differences in luciferase measurements between VEGF-2 and VEGF-C plates do not reflect a variable involving transfection efficiency that could account for the differences in VEGF-2 versus VEGF-C protein expression and secretion that I reported in all three of my previous declarations. In fact, the opposite is true. The luciferase data serves to validate the experimental design. The luciferase measurements were the same order of magnitude at each time point and indicate successful transfection of cells in both the VEGF-2 and the VEGF-C co-transfection experiments. The luciferase activity was abundant and measurable for both the VEGF-2 and VEGF-C co-transfections, whereas in the immunoprecipitation experiments, VEGF-2 has always been unmeasurable, while VEGF-C has always been easily measured. Thus, the transfection and cell culturing techniques are all sound. The "problem" is that the cells cannot express and secrete the VEGF-2 encoded by the VEGF-2 cDNA as taught in the opposed application.

5.2 Even so, for this declaration, I adjusted the immunoprecipitation experiments for *the benefit of the VEGF-2 transfections*, based on the luciferase data. Specifically, I presumed that the luciferase measurements provided an indication of the recombinant protein making capacity of the transfected cells. Based on this assumption, the

VEGF-C cells were making more recombinant protein than the VEGF-2 cells in these particular transfections. To compensate for the apparent difference, I used larger volumes of conditioned media from the VEGF-2 cells than from the VEGF-C cells for the immunoprecipitation. The volumes selected were as follows:

Culture Period	Volume of CM from cells transfected with VEGF-2(HGS)/pcDNA1	Volume of CM from cells transfected with VEGF-C/pcDNA1	Volume of CM from mock transfected cells
24 hours	690 microliters	450 microliters	1000 microliters
48 hours	235 microliters	125 microliters	1000 microliters
74 hours	265 microliters	180 microliters	1000 microliters

5.3. The relative amounts of conditioned media used were inversely proportional to the luciferase measurements. In each instance, more conditioned media from the VEGF-2 cells was used than from the VEGF-C cells to compensate for the lower production of recombinant (luciferase) protein in these cells. (If the VEGF-2 cells were secreting less protein per microliter of conditioned media, the use of larger sample sizes would compensate.)

5.4 No VEGF-2 protein was detected in the conditioned media from the cells transfected with the VEGF-2(HGS)/pcDNA1 construct at any of the time points tested, over a time period of 74 hours (Figure 2, Lanes 1, 4 and 7; see Exhibit KA-2 hereto. The only bands visible in the VEGF-2 lanes of the gel are also visible in the "mock" lanes that have no VEGF-2 construct). In contrast, VEGF-C protein was effectively expressed and secreted by cells transfected with a vector encoding the full length VEGF-C. The different forms of VEGF-C immunoprecipitated from the conditioned medium correspond to partially and fully processed forms of VEGF-C (Figure 2, Lanes 2, 5 and 8. See bands corresponding approximately to the 30, 21.5, and 14.3 size markers). These results are in agreement with the results reported in OKA1, OKA2 and OKA3, and provide still further evidence that VEGF-2 as taught in the opposed application cannot be expressed and secreted by cells.

5.5 As Figure 2 shows, cells do not express and secrete VEGF-2, no matter what time point is used to terminate the experiment. Using extra conditioned media from the VEGF-2 cells fails to change the negative result. Expression of VEGF-C polypeptides, which serves as a positive control, was visible at all time points studied. VEGF-C expression was already visible at 24 hours and was strongly visible at 48 hours and 74 hours.

5.6 The extra experiments that I ran for OKA3 and this declaration demonstrate that all of HGS's criticisms of my earlier experiments were meritless. The protein expression and secretion data reported in OKA3 and the present declaration is essentially identical, indicating that transfection efficiency, time course, and any other miscellaneous factors causing minor fluctuations in recombinant protein production from one experiment to another do not alter the end result. The data reported herein confirms the conclusions of OKA1, OKA2, and OKA3, namely, that cells cannot express and secrete VEGF-2 as taught in the opposed application.

IV. CONCLUDING REMARKS

6.1 The protein expression and secretion studies I report herein were designed to address any criticisms made by HGS with regard to experimental design credibility. The results demonstrate several key points. First, VEGF-2 as taught in the opposed application cannot be expressed and secreted by cells. The data clearly establishes the failure of VEGF-2 to be expressed and secreted at multiple time points over a period of 74 hours (Each of these time points was sufficient to observe expression and secretion of the VEGF-C positive control run under the same experimental conditions). Second, transfection efficiency experiments reported herein rule out the possibility that absence of expression of VEGF-2 was due to insufficient amounts of the VEGF-2 expression vector being introduced into the cells. Even if the minor variations in transfection efficiency could contribute to variations in protein expression and secretion, this phenomenon was accounted for in the immunoprecipitation experiments reported herein by increasing the volumes of the VEGF-2 conditioned medium that were used in the immunoprecipitations. Thus, transfection efficiency, expression and secretion time course, and all other meaningful variables have all been accounted for in this declaration. This declaration,

and my three earlier declarations, all demonstrate that cells cannot express and secrete VEGF-2 as taught in the opposed application.

AND I MAKE this solemn declaration by virtue of the Statutory Declarations Act 1959, and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true in every particular.

DECLARED at Helsinki, Finland

This 14th day of August, 2002

Kari Alitalo

BEFORE ME

(Signature of Notary Public)
HANS SEVELIUS
Notary Public
14.08.2002

HELSINGIN MAISTRAATTI
REGISTRATEN I HELSINGFORS

AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent Application Serial No. 696764 by Human Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by Ludwig Institute for Cancer Research

THIS IS Exhibit KA-1

referred to in the Statutory Declaration

of Kari Alitalo

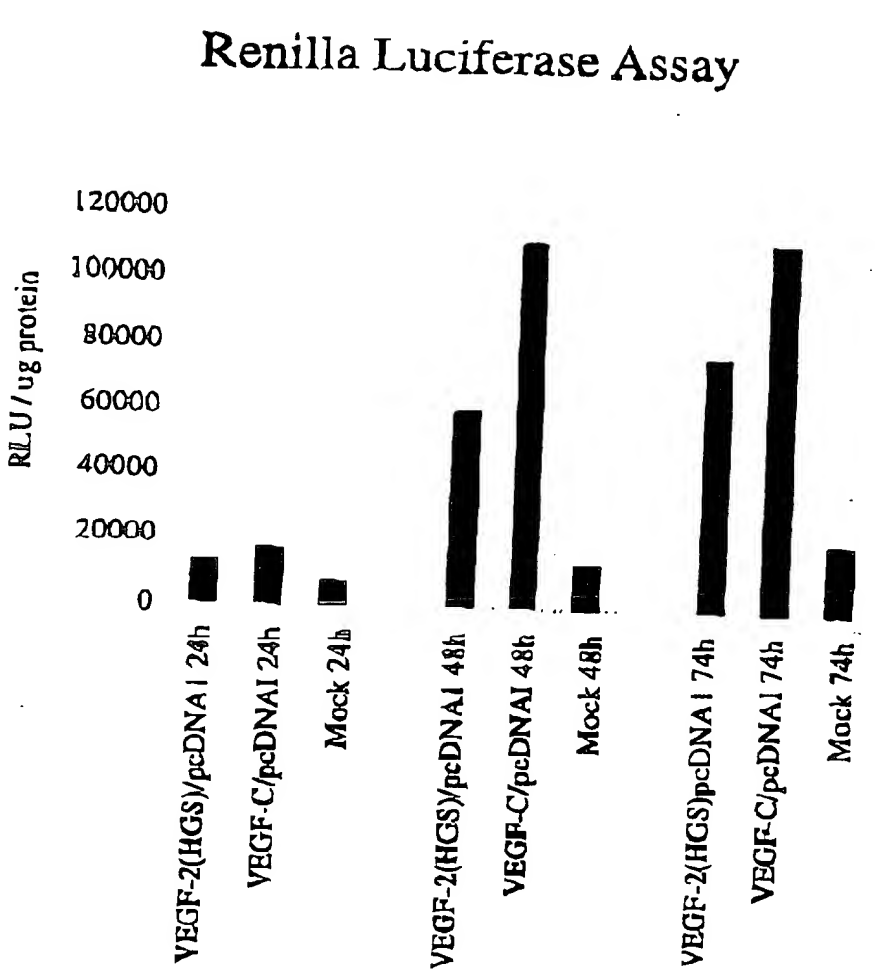
made before me

DATED this 14th Day of August 2002

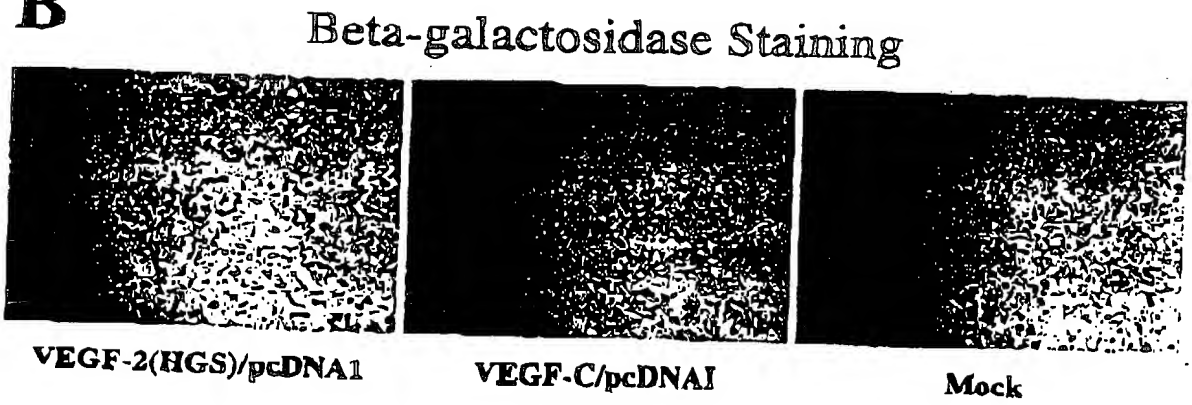
(Signature of Notary Public)
HANS SEVELIUS
Notary Public

HELSINGIN MAISTRAATTI
MAGISTRATEN I HELSINGFORS

A


Renilla Luciferase Assay
RLU / ug protein
120000
100000
80000
60000
40000
20000
0
VEGF-2(HGS)/pcDNA I 24h
VEGF-C/pcDNAI 24h
Mock 24h
VEGF-2(HGS)/pcDNA I 48h
VEGF-C/pcDNAI 48h
Mock 48h
VEGF-2(HGS)pcDNA I 74h
VEGF-C/pcDNAI 74h
Mock 74h

B


Beta-galactosidase Staining
VEGF-2(HGS)/pcDNA1
VEGF-C/pcDNAI
Mock

AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent Application Serial No. 696764 by Human Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by Ludwig Institute for Cancer Research

THIS IS Exhibit KA-2

referred to in the Statutory Declaration

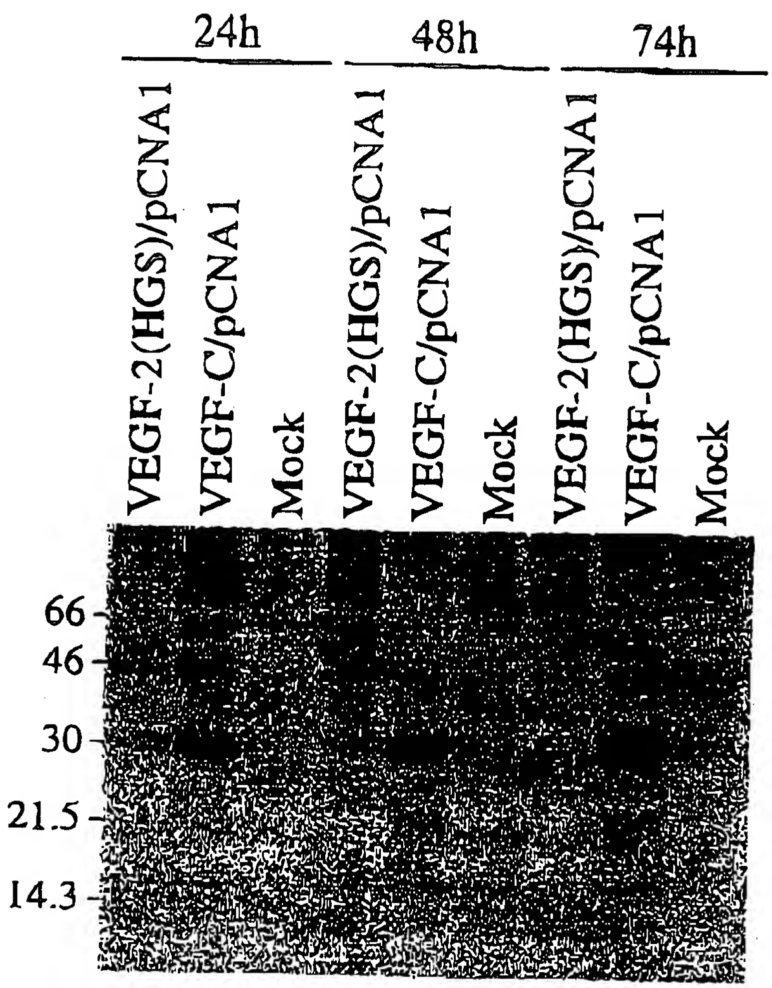
of Kari Alitalo

made before me

DATED this 14th Day of August 2002

(Signature of Notary Public)
HANS SEVELIUS
Notary Public

HELSINGIN MAISTRAATTI
HELSINGFORS


24h
48h
74h
VEGF-2(HGS)/pCNA1
VEGF-C/pCNA1
Mock
VEGF-2(HGS)/pCNA1
VEGF-C/pCNA1
Mock
VEGF-2(HGS)/pCNA1
VEGF-C/pCNA1
Mock
66
46
30
21.5
14.3

Figure

AUSTRALIA

Patents Act 1990

IN THE MATTER OF Australian Patent Application Serial No. 696764 by Human Genome Sciences, Inc.

-and-

IN THE MATTER OF Opposition thereto by Ludwig Institute for Cancer Research

STATUTORY DECLARATION

I, Peter Adrian Walton Rogers of the Department of Obstetrics and Gynaecology, Monash University, Clayton, Victoria, Australia, do solemnly and sincerely declare as follows:

I. INTRODUCTION

1.1 Ludwig Institute for Cancer Research ("Ludwig Institute") has asked for my services as a scientific expert in connection with Ludwig Institute's opposition to the issuance of an Australian patent to Human Genome Sciences, Inc., ("HGS") based on HGS's Australian Patent Application No. 696764. The patent application relates generally to an isolated polynucleotide and protein for an alleged novel vascular endothelial growth factor called "Vascular Endothelial Growth Factor 2" ("VEGF2").

1.2 The first evidence that I provided in the opposition proceeding was a declaration from February 2000 (hereinafter referred to as "OPR1" (Opponent, Peter Rogers, 1st Declaration)). I am aware that Ludwig Institute has also asked Francis John Ballard and Kari Alitalo to serve as scientific experts in this matter, and that they have provided declarations as well.

1.3 In answer to Ludwig Institute's initial evidentiary submission, HGS filed declarations from six scientists, John Stanley Mattick (hereinafter "AJM1"(Applicant, John Mattick, 1st Declaration)), Jennifer Ruth Gamble ("AJG1"), Nicholas Kim Hayward ("ANH1"), Thomas Rapoport ("ATR1"), Stuart Aaronson ("ASA1"), and Susan Power ("ASP1").

1.4 In reply to HGS's declarations, Ludwig filed second declarations from Dr. Alitalo (OKA2), Dr. Ballard (OJB2), and me (OPR2). HGS then obtained permission to file still further evidence, in the form of supplemental declarations by Dr. Power (ASP2), Dr. Hayward (ANH2), and Dr. Aaronson (ASA2). Ludwig Institute asked me to review these most recent declarations filed by HGS and evaluate them in the context of all of the documents that have been filed in this proceeding. Ludwig Institute has also asked me to review declarations of Dr. Ballard and Dr. Alitalo that were prepared in response to ASP2, ANH2, and ASA2.

1.5 I have reviewed all of the foregoing declarations, including the third declarations of Drs. Ballard and Alitalo (OJB3 and OKA3) that were filed in July, 2002, and the fourth declaration of Dr. Alitalo (OKA4) that I understand will be filed in August, 2002.

1.6 I hereby reaffirm my understanding that I have an overriding duty to the Patent Office (and to any Australian Federal Court that should review the Patent Office decision) to provide objective scientific analysis that I believe to be truthful. I hereby affirm that, to the best of my knowledge and belief, factual statements herein are true and opinion statements herein represent my objective scientific opinion and analysis.

II. AFFIRMANCE OF PRIOR DECLARATIONS

2.1 Unless I specifically state otherwise below, I affirm the facts and opinions expressed in my prior declarations (i.e., in OPR1 and OPR2). Nothing in HGS's evidentiary declarations causes me to change the opinions embodied in my earlier declarations.

2.2 In fact, HGS did not specifically dispute my second declaration in the further evidence that they submitted. Whereas my earlier declarations were directed to numerous problems with the opposed application and the HGS evidence in answer, including lack of novelty, inadequate and incomplete description of the invention, hindsight vision by

the HGS declarants, lack of any evidence of VEGF2 biological activity, errors and omissions in the application, and indefinite terminology, the further evidence filed by HGS is primarily directed to the narrower question of the validity and relevance of experiments by Dr. Power and Dr. Alitalo reported in their respective declarations. Therefore, I shall confine most of my remarks herein to those issues, even though I continue to believe that the many problems with the opposed application, enumerated in my earlier declarations, continue to exist.

III. AGREEMENT WITH DR. BALLARD'S THIRD DECLARATION

3.1 I have read the third declaration of Dr. Ballard and I agree with the opinions expressed by Dr. Ballard in his declaration. I hereby incorporate the analysis therein as my own by refcrence.

IV. THE EXPERIMENTS REPORTED IN DR. ALITALO'S DECLARATIONS ARE SOUND, AND THE CRITICISMS OF THEM ARE MERITLESS.

4.1 I have read all four of the declarations of Dr. Kari Alitalo that were prepared in respect of this matter, including the most recent Alitalo declarations (OKA3 and OKA4) prepared in response to the latest declarations filed by HGS.

4.2 I continue to hold the opinion, expressed in OPR2, that the Alitalo experiments were appropriately designed to evaluate the teachings of the opposed application (OPR2 at 2.2, 2.5). The opposed application teaches that VEGF2 is about 350 amino acids of which approximately the first 24 amino acids are likely to be leader sequence such that the mature protein comprises 326 amino acids. (Opposed application at p. 5. See also Figure 1 and sequences.) Dr. Alitalo's experiments remain the only experimental evidence in this proceeding directed to the question of whether VEGF2, as taught in the opposed application, is expressed and secreted by cells. The opposed application has no evidence of successful expression of mature VEGF2, as discussed extensively in my first declaration (OPR1 at 7.6 and sections concerning Insufficiency and Fair Basis). The experimental evidence offered by Dr. Power is not based on the opposed application, as explained in my second declaration (OPR2 at 2.5), in Dr. Ballard's third declaration (OPR3 at 6.1-7.4), and below in greater detail.

4.3 Through four independent sets of experiments, Dr. Alitalo has repeatedly found and reported that VEGF2 expression and secretion as taught in the application does not occur in cells. His second, third, and fourth sets of experiments included modifications to address criticisms raised by HGS declarants. I agree with Dr. Alitalo and Dr. Ballard that the OKA3 and OKA4 experiments contained appropriate controls for the variables questioned by HGS in its second series of declarations, namely, transfection efficiency and time course.

4.4 As explained repeatedly in Ludwig Institute's evidence and admitted by the HGS declarants in their first series of declarations, the VEGF2 of the opposed application is *incomplete* (OPR2 at 4.1, 4.3-4.6). It is missing about 69 codons, including the codons for the signal sequence. These defects were not reported in the opposed application, but were discovered in subsequent years of research. However, these acknowledged defects provide one scientific explanation for why Dr. Alitalo repeatedly finds that VEGF2 as taught in the opposed application cannot be expressed and secreted. In my opinion, the criticisms raised by HGS through its declarations are little more than a smokescreen to distract from the plain truth that the teachings in the opposed application relating to VEGF2 are incomplete. The lack of substance behind the HGS criticisms is illustrated by the willingness of the HGS declarants to espouse inconsistent positions. (See detailed discussion in OJB3, Section IV.) For example, Dr. Hayward first opined that Dr. Alitalo's experiments were unreliable because of inconsistencies in expression and/or processing by different cell lines used for VEGF2 expression studies. (See ANH1 at 5.5.) After Dr. Alitalo provided additional controls and explained that this criticism was without merit, Dr. Hayward asks the Patent Office to believe that "By March 1994 I was aware that any given host cell would possess the proteolytic enzymes and cellular machinery to naturally process a protein such as VEGF-2 to its mature form." (ANH2 at 1.7) If Dr. Hayward were aware as early as 1994 that any cell would work, then why was he criticizing Dr. Alitalo's choice of cells in his ANH1 declaration?

4.5 Most of HGS's criticisms of Dr. Alitalo's work have been directed to whether his experiments contain adequate controls. With almost any experimental summary, a scientist with general knowledge in a field can identify some parameters for which the experimenter did not report controls. At the same time, such a scientist is also familiar with which aspects of an experiment are routine or uncontroversial, and which are more critical to the variable being tested. In my opinion, none of the controls on which the HGS experts focus are serious experimental omissions, if indeed Dr. Alitalo omitted them at all.

4.6 In any event, it is clear to me from reading OKA3 and OKA4 that Dr. Alitalo has run his experiments twice more, with explicit controls for transfection efficiency and time course, the two factors that were the subject of HGS's newest declarations. Dr. Alitalo's results show that these factors are not affecting his results (OKA3 at 6.1; OKA4 at 6.1). Dr. Alitalo's experiments have always been designed to compare expression of VEGF2 as taught in the opposed application with a true, full length VEGF-C cDNA, which can be viewed as a "positive control." The experiments have also included "mock" transfected cells as a negative control. The only meaningful variable in Dr. Alitalo's experiments was the identity of the insert in the expression vector (VEGF2 or VEGF-C). The VEGF-C "positive control" insert worked just fine, but the VEGF2 as taught in the opposed application failed, demonstrating that cells cannot express and secrete VEGF2 as taught in the opposed application.

V. DR. POWER'S DECLARATION

5.1 Dr. Alitalo and Dr. Ballard have explained in their declarations, and I agree, that the experiments by Dr. Power are not based on the teachings of the opposed application (OJB3 at 6.1-7.4; OKA3 at 2.1). I also agree with their conclusion that her results -- "a broad band resolving at approximately 30 kDa" on a gel (see ASP2 at 31) -- have no apparent relevance to the teachings of the opposed application. (See, e.g., OJB3 at 6.1-7.4; OKA3 at 5.1-5.7.) The opposed application teaches that mature VEGF2 comprises 326 amino acids of the VEGF2 sequence taught in the application. Those 326 amino acids have a combined, calculated theoretical molecular weight (for "mature VEGF2") of about 36.8 kD, ignoring the possibility of post-translational modifications such as glycosylation that might increase the observed molecular weight.[1] This calculated weight is in reasonable agreement with the observed molecular weight of the two polypeptides reportedly produced by "in vitro transcription/translation" in Example 2 of the opposed application, which were reportedly 38-40 kD for "the full length VEGF2 cDNA" and 36-38 kD for a polypeptide reportedly missing 36 amino acids from the C terminus. The 30 kD molecular weight reported by Dr. Power in

[1] Of course, the notion of an observed molecular weight is theoretical only, since that would require cells to express and secrete mature VEGF2 as taught in the application, and Dr. Alitalo has shown that this does not happen.

her experiments has no apparent relevance to the teachings in the opposed application regarding VEGF2.

5.2 To explain this discrepancy in a different way, I would say that the skilled person, in attempting to express VEGF2 as taught in the opposed application, would have been looking for a polypeptide of approximately the full length as taught by HGS. That person would have expected to produce a mature VEGF2 of about 37 kD based on calculated molecular weight and based on the limited guidance provided by Example 2 of the opposed application. For these and other reasons, it is my opinion that Dr. Power's experiments should not be accepted as relevant to the opposed application. Even if, for argument's sake, Dr Power's results are accepted for some purpose, I do not believe that a scientist in 1994, without the benefit of what is now known about VEGF-C, would have identified anything other than the 326 amino acid "mature VEGF2" polypeptide from the application itself, and would not have had any reason to investigate anything other than the 326 amino acid polypeptide.

5.3 Dr. Power's experimental approach to solving defects in the opposed application was clearly developed with the benefit of hindsight. (See, e.g., OPR2 at 4.24 and 4.54-4.71.) Dr. Alitalo's work shows that a scientist in 1994 attempting to express the VEGF2 sequence by following the teachings of the opposed application would have been unsuccessful, e.g., because as extensively explained in the Ludwig Institute's evidence and sometimes admitted by HGS declarants, the VEGF2 of the opposed application is incomplete. (OPR2 at 4.1 and 4.3-4.6) It is missing about 69 codons, including the codons for the signal sequence. That scientist would have then needed further experimentation to identify the source of the problem or problems and determining the solution or solutions to those problems. However, without the benefit of hindsight that we now enjoy, that scientist would not have known that the failure was due, at least in part, to a missing signal peptide and other N-terminal sequences.

5.4 The absence of a signal sequence would not, in my opinion, have been the only possibility nor would it have been the first possibility as to the source of the problem. In fact, I believe the scientist would have been comforted and guided by the express statements in the opposed application that the VEGF2 sequence as taught therein includes a signal sequence, and so would have concentrated on the numerous other possible problems in expressing a novel, as yet uncharacterized human cDNA. These possibilities

include problems associated with the vector (such as absence of controlling elements such as promoter sequence, enhancer sequence), problems associated with the host cells (such as inadequate growth and proteolysis), problems of incompatibility of the host cells and the vector or the encoded protein, problems associated with the induction of expression (such as inactive or insufficient inducing agent), problems associated with the coding sequence (such as the presence of premature termination codon), problems associated with the detection system (such as low titre of an antibody for antibody detection, lack of activity for an activity-based detection, defective label for label-based detection), problems associated with degradation, and so on. I discussed these issues extensively in my second declaration. (See OPR2, sections regarding Fair Basis and Insufficiency.)

5.5 It is interesting to observe that when HGS discovered that the complete VEGF2 cDNA contains approximately an additional 69 codons, meaning that VEGF2 as taught in the opposed application is incomplete, HGS considered the discovery to be significant enough to file a second series of patent applications on the 419 amino acid form of VEGF2. (See Documents D44-D46; OPR1 at 1.5.1-1.5.2). In the second series of applications, HGS teaches that the immature and "mature" forms of VEGF2 are substantially longer than what was taught in the opposed application (419 and 396 amino acids, respectively).)See Documents D43 and D44 at p. 7, last paragraph; OPR1 at 4.11.1.1.) Of course, in this opposition proceeding, HGS and/or Dr. Power can design some experiments focused directly on the problem of the missing signal peptide, but only through the benefit of hindsight knowledge that VEGF2 as taught in the opposed application is missing the signal peptide.

VI. DR. ALITALO'S "FAILURE" TO USE A HETEROLOGOUS SIGNAL SEQUENCE.

6.1 Drs. Hayward and Aaronson argued that Dr. Alitalo's experiments are flawed because in Dr. Alitalo's declarations, he did not report on the effect of attaching a heterologous signal sequence to the 350 amino acid VEGF2 sequence. (See, e.g., ANH2 at 1.5, 1.6, 1.10; ASA2 at 5, 6-10, 13-22.) Dr. Power's experiments involve heterologous signal peptides.

6.2 As explained in the preceding section and by Dr. Ballard in his third declaration (e.g., OJB3 at 6.1-7.4), I disagree with the Hayward/Aaronson analysis and

disagree with any suggestion that Dr. Power's experimental approach is a fair representation of anything taught in the opposed application. The opposed application teaches that VEGF2 already has a signal sequence. (See, e.g., opposed application at p. 5, last paragraph.) It is illogical to attach a heterologous signal sequence to a sequence which already has a signal sequence. Solving the riddle of VEGF2 expression would have required a scientist to ignore the specific teachings regarding VEGF2 that are found in the opposed application and arrive at a solution that is completely at odds with those teachings. As extensively explained in Ludwig Institute's second evidentiary submission, the opposed application does not teach to perform the experiments urged by HGS. [OJB2 at 2.2, 3.19-3.24, 6.1-6.6.; OPR2 at 4.4-4.6, 4.12, 4.14-4.53.]

6.3 In my opinion, Dr. Alitalo's experiments were properly designed to test the opposed application's teachings, which are that full length VEGF2 comprises 350 amino acids, of which approximately the first 24 amino acids are a signal sequence. (See, e.g., opposed application at pp. 4-5). These were the specific teachings to the public about the subject matter of the opposed application. The experiments urged by HGS are a form of revisionist history - an attempt to fix a defective patent application by hindsight knowledge of a protein learned from publications by Dr. Alitalo and others. [See, e.g., Documents D70-D73.] The question as I understand it is not whether a person in year 2001 or 2002, knowing what Dr. Alitalo and others have taught the public about the VEGF-C gene and protein, can apply that knowledge and succeed at expressing some portion of the VEGF2 sequence taught in HGS's 1994 patent application. The question is whether the teachings of the application were accurate and placed the public in possession of a complete and working invention. Dr. Alitalo tested the teachings of the opposed application and showed that the answer to this question is no.

VII. CONTINUED APPLICATION OF A SCIENTIFIC DOUBLE STANDARD BY HGS.

7.1 In Section III of OJB3, Dr. Ballard explains in detail one of the ironies of this opposition proceeding. The opposed patent application filed by HGS contains very little in the form of experimental or evidentiary support, and many of the experiments that were described therein have been shown to be defective, as explained in my first declaration. (See, e.g., OPR1 at 4.13-4.13.3.) Still, HGS's declarants seem perfectly content believing

every aspect of the opposed application, including aspects involving treatment of a huge variety of human diseases and ailments. At the same time that HGS's declarants adopt a position of faith with respect to the unsupported teachings of the opposed application, the same HGS declarants have urged that the Patent Office adopt very stringent criteria in this opposition proceeding for evaluation of scientific data presented by Ludwig Institute in its opposition. (See ANH2 and ASA2 declarations.)

7.2 Whether evaluating a patent application involving molecular biology or evaluating a sworn declaration involving molecular biology, a trained scientist should be applying the same scientific criteria. Scientifically, it makes no sense to believe one set of conclusions about VEGF2 that are unsupported by scientific evidence, even if they are written in a patent application, and to ignore scientific evidence that clearly contradicts the conclusions. Yet that is exactly what one finds upon examination of the HGS declarations. (See, e.g., OPR2, Appendix II. See also ANH2 and ASA2.)

7.3 For example, Dr. Hayward and Dr. Aaronson have urged that Dr. Alitalo's experiments should be given no weight, because of alleged defects in his selection or reporting of positive and negative controls. (See, e.g., ANH2 at 1.5; ASA2 at 5, 12, and 23.) However, the experiments reported in the opposed application clearly fail to report use of appropriate controls. (See OJB3 at 3.3-3.5) The statements in the opposed application regarding biological activity and treatment of diseases are unsupported by any reported experiments whatsoever, with or without controls. (OJB3 at 3.6) There is no scientific justification for placing faith in the teachings of the opposed application, especially when confronted with the evidence provided by Dr. Alitalo that the teachings in the opposed application are defective. Yet, that is exactly the position that the HGS declarants continue to adhere to. For example, Dr. Aaronson expresses the opinion that one familiar with the molecular biology of growth factors and "equipped with the HGS patent specification would recognize that the 350 amino acid polypeptide is a secreted growth factor . . . ." (ASA2 at 10.) Dr. Aaronson characterizes this as a "literal teaching of the HGS patent specification which describes VEGF-2 as a secreted growth factor . . . ." (ASA2 at 11.) These are, at best, statements of faith, because according to the scientific standards that Dr. Aaronson articulated for evaluating the Alitalo experiments, the skilled molecular biologist *should* conclude that the opposed application "fail[s] to provide any meaningful information regarding the expression, processing and secretion of VEGF-2" or its use as a "growth factor" for any purpose. (Compare ASA2 at 5 and 12.)

VIII. CONCLUDING REMARKS

8.1 Having examined the opposed application and all of the declarations that have been submitted by both parties, including the third and fourth Alitalo declarations, the following conclusions are self evident: (1) Dr. Power performed experiments involving VEGF2 subject matter, but her experiments were not based on, and did not test, the direct teachings in the application, i.e., whether VEGF2 of about 350 amino acids is expressed and secreted by cells. (2) Dr. Alitalo's experiments were based on these teachings of the opposed application, and they were refined over and over to address every criticism that a group of HGS experts could think of. Dr. Alitalo's results showed repeatedly that expression and secretion of VEGF2 does not occur in the manner taught by the opposed application. (3) HGS asked scientists other than Dr. Power to criticize Dr. Alitalo's work, but not actually run Dr. Alitalo's experiments themselves, or run any other experiments based on the explicit teachings of the application, or report the results of such tests. As a result, the only experimental evidence testing the teachings of the opposed application is the evidence reported by Dr. Alitalo.

AND I MAKE this solemn declaration by virtue of the Statutory Declarations Act 1959, and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true in every particular.

DECLARED at Monash Medical Centre, Vic

This 16 day of August, 2002

Before me:

Peter Adrian Walton Rogers

(Signature of Witness) (Medical Doctor)

American Type Culture Collection

12301 Parklawn Drive o Rockville, MD 20852 USA o Telephone: (301)231-5520 Telex: 898-055 ATCCNORTH o FAX: 301-770-2537

PF12

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Human Genome Science, Inc.
Attention: Craig A. Rosen, Ph.D.
9820 Medical Center Drive, Suite 300
Rockville, MD 20850

Deposited on Behalf of: Human Genome Science, Inc.

Identification Reference by Depositor:	ATCC Designation
DNA Plasmid, 148,362	75696
DNA Plasmid, 23,613	75697
DNA Plasmid, 182,618	75698 - PF112
DNA Plasmid, 161,797	75699 PF110
DNA Plasmid, 721	75700
DNA Plasmid, 1968	75701

The deposits were accompanied by: __ a scientific description __ a proposed taxonomic description indicated above.

The deposits were received March 4, 1994 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested March 10, 1994. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:
for B.A. Brandon Date: March 14, 1994
Bobbie A. Brandon, Head, ATCC Patent Depository
cc: Greg Ferraro

PF 112 P1

American Type Culture Collection

12301 Parklawn Drive o Rockville, MD 20852 USA o Telephone: (301)231-5520 Telex: 898-055 ATCCNORTH o FAX: 301-770-2587

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Human Genome Sciences, Inc.
Attention: Robert H. Benson
9410 Key West Avenue
Rockville, MD 20850

Deposited on Behalf of: Human Genome Sciences, Inc.

Identification Reference by Depositor:	ATCC Designation
DNA Plasmid, 567,663 (HGS Docket PF112P1	97149
DNA Plasmid, 573,374 (HGS Docket PF171)	97150

The deposits were accompanied by: _ a scientific description _ a proposed taxonomic description indicated above.

The deposits were received May 12, 1995 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested May 18, 1995. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Annette L. Bade, Director, Patent Depository

Date: May 22, 1995

cc: Greg D. Ferraro

Express Mail No.:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Rosen *et al.*

Application No.: 08/465,968

Filed: June 6, 1995

For: Human Vascular Endothelial Growth Factor 2

Confirmation No.: 2539

Group Art Unit: 1647

Examiner: C. Saoud

Attorney Docket No.: PF112P1

#32
B.9.)
2/11/03

DECLARATION OF STUART AARONSON
UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, STUART AARONSON, do declare that:

1. I am currently the Director of the Derald H. Ruttenberg Cancer Center for the Mount Sinai Medical Center in New York, New York. Since the 1970s, my research has focused on growth factors and their role in tumorigenesis and cancer as evidenced by my curriculum vitae, attached hereto as Exhibit A, which lists the publications that I have authored or co-authored. My research in the area of the molecular biology of growth factors and their receptors, including keratinocyte growth factor, fibroblast growth factor and vascular endothelial growth factor, has encompassed mammalian models of tumorigenesis, including human tumors.

2. I have been asked by the Patent Attorneys representing Human Genome Sciences ("HGS") to review the specification of U.S. application serial no. 08/465,968, filed June 6, 1995, ("the '968 patent specification") and the claims pending in connection with the '968 patent application. I have been asked to provide my comments and opinions as to what the patent specification would provide to one familiar with the molecular biology of growth factors, *e.g.*, a post doctorate or

Ph.D. candidate in a research laboratory, as of June 1995. For purposes of this analysis, I considered not only what I knew and appreciated at the relevant time, but what was expected to be known by graduate students and postdoctoral fellows who were in my laboratory at the relevant time.

3. I have reviewed and analyzed the polynucleotide and amino acid sequence identified by HGS to encode the human VEGF-2 protein, as set forth in Figure 1 of the '968 patent specification. I have also reviewed and analyzed the characterization of the sequence of VEGF-2 as having a high level of homology with other members of the PDGF/VEGF family (*see, e.g.*, Figure 2 of '968 patent specification). The '968 patent specification describes, but is not limited to, the characterization of the human VEGF-2 protein as structurally related to the PDGF/VEGF family. The '968 patent specification reports that at the amino acid level, VEGF-2 exhibits the highest homology to vascular endothelial growth factor (30% identity), followed by PDGF alpha (23%) and PDGF beta (22%) (*see* the '968 patent specification at page 7, line 25 to page 8, line 3). The '968 patent specification describes that amino acid residues 85 to 165 of VEGF-2 (as shown in SEQ ID NO:2) contain the eight cysteine residues which are strictly conserved among all known members of the PDGF/VEGF family, and in addition, also contains the fourteen amino acid signature motif, PXCVXXXRCXGCCN, found in all members of the PDGF/VEGF family (*see* the '968 patent specification at page 8, lines 4-8 and the boxed areas of Figure 2). As set out in the '968 patent specification, the retention of these conserved structural characteristics by members of the PDGF/VEGF family of growth factors is critical to the proteins' capability to exert their growth promoting or mitogenic effects (*see* the '968 patent specification at page 8, lines 44-8 and page 8, lines 12-18).

4. The pending claims of the '968 patent specification are directed to VEGF-2 polypeptides having at least amino acid residues 85 to 165 of SEQ ID NO:2, methods of making such polypeptides, polynucleotides that encode such polypeptides, and vectors and host cells comprising such polynucleotides. The pending claims also relate to methods of stimulating proliferation of endothelial cells in a patient comprising administering polypeptides having at least amino acid residues 85 to 165 of SEQ ID NO:2.

Prior Art Demonstrates Retention of a Region Spanning Eight Cysteine Residues Strictly Conserved Among Members of the PDGF/VEGF Family Is Required To Maintain Biological Activity In PDGF

5. I have also been asked to review publications available as of June 1995 discussing the importance of the conserved motif of eight conserved cysteines in the known members of the PDGF/VEGF family. In particular, I have been asked to review Hannink et al., 1986, Molecular and Cellular Biology 6(4):1304-1314 (attached hereto as Exhibit C, "Hannink"), and comment on what the publication would provide to a molecular biologist familiar with the biology of growth factors. Hannink characterizes the regions of PDGF that are conserved among members of the PDGF/VEGF family and reports experimental results correlating the presence of a structural motif of eight cysteine residues strictly conserved among the members of the PDGF/VEGF family with retention of biological activity.

6. Hannink reports that C-terminal deletion analysis of the v-*sis* gene which encodes chain B of PDGF has demonstrated that amino acid residues 112 to 214 of PDGF (as shown in Figure 6 of Hannink) are sufficient for retention of PDGF activity, *i.e.*, mitogenesis and transformation (*see* Hannink at page 1304, col. 2 to page 1305, col. 1; page 1311, col. 2; and Figure 6). Amino acid residues 112 to 214 define a 103 amino acid region that spans the structural motif of eight cysteine residues that is strictly conserved amongst the known members of the PDGF/VEGF family (*see* Hannink at Figure 6 as compared to the '968 patent specification at Figure 2).

7. Because VEGF-2, as characterized in the '968 patent specification, is a new member of the PDGF/VEGF family which contains the conserved structural motif characterized by eight strictly conserved cysteine residues which confer biological activity, a molecular biologist familiar with the molecular biology of growth factors would reasonably expect the strict conservation of the eight cysteine residues in VEGF-2 to be a basis for the retention of VEGF-2 biological activity, as described in the '968 patent specification. The expectation that amino acids 85 to 165 of SEQ ID NO:2 would be critical to maintain VEGF-2 activity is consistent with was known about other PDGF/VEGF family members.

8. Thus, as of the filing date of the '968 patent specification, not only was it known that members of the PDGF/VEGF family contained a structural motif that was strictly conserved among its members (*i.e.*, a region spanning eight strictly conserved cysteine residues); it was also known that retention of the region spanning the conserved cysteine residues is critical to maintain biological activity.

The HGS Patent Specification Provides Sufficient Information Such That A Molecular Biologist Recognizes The Region of VEGF-2 Important For Maintaining VEGF-2 Activity

9. Based on the characterization of the VEGF-2 protein set forth in the '968 patent specification, one would recognize that the protein was a member of the PDGF/VEGF family of growth factors. As a member of the PDGF/VEGF family of growth factors, one would recognize that in order to maintain biological activity of VEGF-2, those highly conserved motifs and signature sequences should be retained. As provided by the '968 patent specification, the highly conserved motifs and signature sequences of the PDGF/VEGF family include: the eight conserved cysteine residues and the family signature sequence, PXCVXXXRCXGCCN. The '968 patent specification provides that the conservation of the conserved motifs and signature sequences are important for the retention of biological activity, such as stimulation of proliferation of endothelial cells (*see* the '968 patent specification at page 8, lines 4 to 18).

10. Further, the '968 patent specification clearly sets forth that one of the biological activities of VEGF-2 includes stimulation of proliferation of endothelial cells. The '968 patent specification provides that active polypeptides are meant to include any portion of the full length amino acid sequence which have less than the full length amino acid sequence as shown in SEQ ID NO:2, but still contain the eight cysteine residues shown conserved in Figure 2 *and* that such fragments still contain VEGF-2 activity (*see* the '968 patent specification at page 8, lines 12 to 18).

11. The '968 patent specification provides that the eight conserved cysteines of VEGF-2 span residues 85 to 165 of SEQ ID NO:2, as shown in Figure 2. The '968 patent specification further discloses that the region of VEGF-2 containing the eight conserved cysteines is critical to the retention of VEGF-2 activity.

Therefore, I or any other molecular biologist provided with the '968 patent specification would recognize the importance of the conserved motifs (*i.e.*, residues 85 to 165 of SEQ ID NO:2) for maintaining VEGF-2 activity.

Post-Filing Reference Corroborates That The Region Of VEGF-2 Corresponding To The Conserved Motif Of The PDGF/VEGF Family Confers Biological Activity

12. I have been asked to review post-filing reference Alitalo et al., U.S. Patent No. 6,361,946 (attached hereto as Exhibit B, "Alitalo"), and comment on the experimental results described therein. The results reported corroborate the expectation that I or any other molecular biologist familiar with the molecular biology of growth factors would have, based on the '968 patent specification, that a polypeptide having less than the full length sequence of VEGF-2 but retaining amino acid residues 85 to 165 of SEQ ID NO:2 would be required to maintain VEGF-2 activity. Results from Alitalo discussed *infra* confirm that recombinant polypeptides retaining amino acid residues 85 to 165 of VEGF-2 retain VEGF-2 activity, *e.g.*, the ability to autophosphorylate VEGF-2 receptors, stimulate proliferation and migration of endothelial cells, and increase permeability of blood vessels.

13. Alitalo extrapolates from sequence comparisons of members of the VEGF family of polypeptides fragments even smaller than residues 85 to 165 would retain VEGF-2 biological activity (*see* Alitalo at col. 5, lines 30 to 45; col. 48, line 56 to col. 49, line 4). Alitalo explains that the eight conserved cysteine residues of the VEGF family (*e.g.*, residues 85-165 of VEGF-2) define a region of evolutionary significance and would therefore expectedly confer VEGF-2 biological activity (*see* Alitalo at col. 5, lines 34 to 39; col. 48, lines 59 to 64). Indeed, based on the sequence and experimental data described therein, Alitalo postulates that a polypeptide corresponding to as few as amino acid residues 115 to 165 of VEGF-2 would still retain biological activity (*see* Alitalo at col. 5, lines 30 to 42 and col. 48, line 56 to col. 49, line 4).

14. Therefore, the post-filing evidence corroborates that, as explicitly provided by the '968 patent specification, amino acid residues spanning the eight cysteine conserved motif of the PDGF/VEGF family, *i.e.*, residues 85 to 165 of SEQ ID

NO:2, are critical to the retention of VEGF-2 activity, such as stimulation of endothelial cell proliferation.

Conclusion

15. In summary, for the above reasons and observations, I believe that I, or any other molecular biologist familiar with growth factors, provided with the '968 patent specification would recognize that the highly conserved motifs and signature sequences of the PDGF/VEGF family, *i.e.*, amino acid residues 85 to 165 of SEQ ID NO:2, are critical to retaining VEGF-2 activity, *e.g.*, the ability to stimulate proliferation of endothelial cells.

16. I declare further that all statements made in this Declaration of my knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

11/15/02
Date

Stuart Aaronson

Exhibit A

6/28/00

CURRICULUM VITAE

Name: Stuart A. Aaronson

Date and Place of Birth: February 28, 1942, Mt. Clemens, Michigan

Citizenship: U.S.A.

Marital Status: Married, three children

Education and Training:

1959-1962	B.S. (Chemistry; summa cum laude), University of California, Berkeley
1962-1966	M.D., University of California Medical School, San Francisco
1965-1966	Fellowship, Dept. of Biochemistry, University of Cambridge, Cambridge, United Kingdom
1966-1967	Intern, Medicine, Moffitt Hospital, San Francisco

Brief Chronology of Employment:

1967-1969	Staff Associate, Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, MD
1969-1970	Senior Staff Fellow, Viral Carcinogenesis Branch
1970-1977	Head, Molecular Biology Section, Viral Carcinogenesis Branch
1977-1993	Chief, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland
1993-	Director, Ruttenberg Cancer Center, Mount Sinai Medical Center, New York, NY & Jane B. and Jack R. Aron, Professor of Neoplastic Diseases

Medical Licenses

New York
Virginia

Honors and Awards:

1962	Phi Beta Kappa
1966	Alpha Omega Alpha
1982	Rhoads Memorial Award
1982	PHS Meritorious Service Medal
1989	Paul Ehrlich Award
1989	PHS Distinguished Service Medal
1990	Milken Award
1991	Chirone Prize
1991	Harvey Lecture

1991 Wadsworth Memorial Foundation Award

Societies:

American Society for Microbiology
American Association for the Advancement of Science
Society for Experimental Biology and Medicine
American Association for Cancer Research, Inc.
American Society for Virology, Inc.

Memberships and Affiliations:

1975-1978 Member, Viral Cancer Program Coordinating Committee
1975-1976 Ad Hoc Member, Experimental Virology Study Section, NIH
1975-1978 Member, Viral Oncology Scientific Advisory Committee for FCRC
1976-1980 Member, Experimental Virology Study Section, NIH
1977- Member, Editorial Board, International Journal of Cancer
1977-1986 Associate Editor, Journal of the National Cancer Institute
1980-1985 Editorial Advisory Board, Biochimica et Biophysica Acta (BBA Reviews on Cancer)
1981- Associate Editor, Cancer Research
1983- Executive Committee, Duke Comprehensive Center, Duke University Medical Center
1984 Mott Selection Committee, General Motors Cancer Research Foundation
1984- Advisory Committee, Maimonides Conferences on Cancer Research
1984-1990 Editorial Board, Virus Research
1984-1987 Scientific Advisory Committee, American Cancer Society
1985-1987 External Scientific Review Committee, Comprehensive Center, The University of Alabama in Birmingham
1985- Editorial Advisory Board, Cancer and Metastasis Reviews
1985- Editorial Board, Cancer Reviews
1985-1989 Councillor, Society for Experimental Biology and Medicine
1985-1990 Extramural Advisory Board, Cancer Center, The University of Arizona
1986 Program Chairman, American Association of Cancer Research
1986 Co-organizer, Princess Takamatsu Symposium
1986- Guest Editor, Japanese Journal of Cancer Research (Gann)
1986- Editorial Board, Environmental and Occupational Health Sciences
1986-1987 Member, Advisory Committee, American Type Culture Collection
1987-1989 Editorial Advisory Board, Molecular Endocrinology
1987- Editorial Board, Oncogene
1988-1989 Advisory Editorial Board, ISI Atlas of Science: Biochemistry
1988- Member, Blood Services Scientific Council, American Red Cross
1989-1991 Editorial Board, Cancer Communications

1989-1992 Editorial Board, The New Biologist
1989 Visiting Professor, University of Texas, San Antonio
1990- Advisory Board, BBA Reviews on Cancer, Biochimica et Biophysica Acta
1990- General Motors Visiting Professor, University of Wisconsin-Madison Medical School
1990- Visiting Professor, Jonsson Comprehensive Cancer Center, University of California, Los Angeles
1992- Editorial Board, Intl. Journal of Oncology
1992- Editorial Board, Oncology Research
1992 Scientific Advisory Board, - Georgetown Univ – Breast Ca SPORE
1993-1995 Editorial Advisory Board, Molecular Aspects of Medicine
1994- International Advisory Board, Tumori
1995-1996 Vice President, Harvey Society
1995- External Scientific Advisory Committee, UCLA Oral Cancer Center
1996-1997 President, Harvey Society
1997-1998 Counselor, Harvey Society
1998 Member, Public Relations and Communications Committee, AACR
1998 Member, The National Neurofibromatosis Foundation Research Advisory Board

Research Interests:

Molecular genetics of cancer; retrovirology; cellular growth regulation by growth factors and their receptors.

Patents:

More than 50 patent applications issued or pending.

Social Security Number:

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Vol. 6, No. 4

Deletions in the C-Terminal Coding Region of the v-*sis* Gene: Dimerization Is Required for Transformation

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Received 10 October 1985/Accepted 20 December 1985

The v-*sis* gene encodes chain B of platelet-derived growth factor. However, this gene codes for additional amino acids at both the N terminus and the C terminus of its gene product which are not present in the amino acid sequence of platelet-derived growth factor. We constructed a series of deletion mutants with deletions in the v-*sis* gene in order to define the C-terminal limit of the v-*sis* gene product which is required for transformation. Deletion mutants of the v-*sis* gene which encoded truncated gene products up to 57 residues shorter than the v-*sis*^{mt} gene product were still able to transform cells. The minimal transforming region of the v-*sis* gene product contained six residues fewer than were present in chain B of platelet-derived growth factor. Only 10 residues, including the sequence Cys-Lys-Cys, separated the smallest transforming gene product from the largest nontransforming gene product. These cysteine residues were also important for dimerization of the v-*sis* gene product, since all of the nontransforming v-*sis* deletions were unable to form dimers when they were analyzed under nonreducing conditions. Our results suggest that there is a strong connection between transformation and dimerization.

The v-*sis* oncogene was isolated as the oncogene of simian sarcoma virus (SSV) (3, 27). SSV acquires cellular genetic information at the expense of a portion of its *env* gene, resulting in the formation of an *env-sis* fused open reading frame (3). This 813-nucleotide open reading frame is potentially capable of encoding a 271-amino acid protein. There are a total of six ATG codons in the *env-sis* open reading frame, and previous work has shown that the first ATG codon is used in the synthesis of the initial translation product (submitted for publication). Located between the second and third ATG codons is a coding region for a signal peptide sequence which directs the cotranslational translocation of the nascent polypeptide chain across the membrane of the rough endoplasmic reticulum (10). Signal sequence cleavage and the addition of an N-linked oligosaccharide to asparagine residue 93 (counting from the N terminus of the predicted initial translation product) occur during translation (22; submitted for publication). (Note that throughout this paper the amino acid number of a residue is its distance from the N terminus of the predicted initial translation product.) Approximately 4 kilodaltons (kDa) is removed from the N terminus of the initial translation product as a result of signal sequence cleavage. The resulting glycoprotein, which is detected as a 28-kDa protein (21) or a 32-kDa protein (submitted for publication), dimerizes soon after crossing the rough endoplasmic reticulum membrane. During transport through the Golgi apparatus to the cell surface, the v-*sis* gene product is proteolytically processed at a basic dipeptide (Lys-Arg at residues 110 and 111), resulting in the formation of a 42-kDa dimer composed of two 20-kDa subunits (21). Further processing of the v-*sis* gene product has been reported to occur at the C terminus, resulting in the formation of a platelet-derived growth factor (PDGF)-related 24-kDa dimer (21). Secretion of a v-*sis*-encoded 17-kDa protein (20) or 20-kDa protein (6) from SSV-transformed cells has also been reported. The secreted form(s) of the v-*sis* gene product also exists as dimers when preparations are analyzed under nonreducing conditions.

The v-*sis* gene encodes chain B of PDGF (4, 16, 29). PDGF consists of a dimer of two subunits (16, 29), although it is not known whether PDGF is a pair of related homodimers or an A-B heterodimer. PDGF is a potent mitogen for cells of mesenchymal origin (24) and stimulates the autophosphorylation of its cell surface receptor (19). Both the intracellular and secreted forms of the v-*sis* gene product possess mitogenic activity for NIH 3T3 cells (2, 6, 14, 20) and the secreted form stimulates the autophosphorylation of the PDGF receptor (6, 14, 20). Dimerization of both the v-*sis* gene product and PDGF is required for mitogenic activity (20). Consistent with this requirement for dimerization is the presence of 10 cysteine residues in the v-*sis* gene product, 9 of which are conserved in the c-*sis* gene product. A comparison of the amino acid sequence data for PDGF with the predicted amino acid sequence of the v-*sis* gene product has shown that the PDGF-homologous region of the v-*sis* gene product extends from residue 112 to residue 220, a region containing 8 of the 10 cysteine residues (15). Therefore, the v-*sis* gene product contains 51 residues at its C terminus which are not present in PDGF. These residues are also present in the predicted amino acid sequence of the full-length c-*sis* gene product. The importance of these C-terminal residues for transformation is not known. A previous report indicated that a truncated 239-amino acid v-*sis* gene product retains biological activity (17). However, this was only a single endpoint and still retained 19 amino acids beyond the PDGF-homologous region.

In this study we determined the C-terminal limit of the biologically active v-*sis* gene product. We constructed a series of mutants with deletions in the v-*sis* gene and assayed the mutant v-*sis* genes for their ability to induce focus formation in NIH 3T3 cells. We found that up to 57 amino acid residues could be removed from the C terminus of the v-*sis* gene product without affecting its transformation potential. An analysis of the gene products encoded by the transforming v-*sis* deletions by immunoprecipitation with anti-PDGF serum suggested that C-terminal processing does not occur. However, a truncated v-*sis* gene product which lacked the C-terminal 67 residues was completely unable

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induce focus formation in NIH 3T3 cells. Likewise, additional C-terminal truncations or an internal deletion in the v-*sis* gene product was unable to transform cells. Our results delimited the minimal transforming region of the v-*sis* gene product to 103 residues, extending from residue 112 to residue 214 (six residues shorter than the PDGF-homologous portion of the v-*sis* gene product). Located within the 10 residues which separated the smallest transforming v-*sis* gene product from the largest nontransforming v-*sis* gene product were two cysteine residues, residues 208 and 210. The inability of the nontransforming v-*sis* gene products to form dimers when preparations were analyzed under nonreducing conditions indicated that these cysteine residues are involved in dimer formation of the v-*sis* gene product. Our results demonstrated that dimer formation is required for transforming ability, as well as the mitogenic activity of the v-*sis* gene product.

MATERIALS AND METHODS

Construction of deletion mutants of the v-*sis* gene. The v-*sis* gene contains a TGA codon in a +1 reading frame located within the *Bst*EII site at nucleotide 711 (counting from the first ATG codon of the *env-sis* open reading frame). This TGA codon was inserted into the correct reading frame of the v-*sis* gene by the manipulations described below. To construct the deleted v-*sis* gene that encoded 239 amino acids (v-sis^{239}), a subclone of the v-*sis* gene in pBR322 (pDD119) was linearized by restriction digestion with *Bst*EII. The 5' overhang was filled in by using the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of the four deoxynucleoside triphosphates. The blunt-ended molecules were ligated by using T4 DNA ligase in the presence of ATP and were used to transform competent *E. coli* C600 cells. The resulting plasmid contained a −1 frameshift at nucleotide 717, putting the TGA codon in the reading frame of the v-*sis* gene. To construct the v-sis^{223} gene, a portion of the blunt-ended molecules described above was further digested with *Sma*I before ligation and transformation. This deletion of 43 nucleotides caused a −1 frameshift, which puts the TGA codon in frame at nucleotide 669. To construct the v-$sis^{177/239}$ gene, a 1,500-base pair *Pvu*II-to-*Hin*dIII fragment containing the 5' portion of the v-*sis* gene was isolated. This fragment was ligated to a 3,500-base pair filled-in *Bst*EII-to-*Hin*dIII fragment containing the 3' end of the v-*sis* gene and the vector portion of pBR322. This resulted in an internal deletion of 183 nucleotides, and the correct reading frame was maintained across the restored *Bst*EII site. This plasmid was used in the construction of the v-sis^{177} gene. Digestion with *Bst*EII followed by fill-in of the 5' overhang and ligation of the blunt-ended molecules resulted in a −1 frameshift, putting the TGA codon in the v-*sis* reading frame. To construct the v-sis^{136} gene, pDD119 was linearized at the *Bgl*II site at nucleotide 403 by digestion with *Bgl*II. The 5' overhang was filled in, and an 8-mer *Xba*I linker, CTCTAGAG, was ligated into the molecules. The ligation mixture was transfected into competent *E. coli* cells, and recombinant molecules containing an *Xba*I site in place of the *Bgl*II site were selected. This resulted in the TAG codon from the linker being placed into the reading frame of the v-*sis* gene . The nucleotide sequences of these deletion mutants of the v-*sis* gene were confirmed by nucleotide sequencing (18).

For the *Bal* 31 deletions, the manipulations described below were performed to obtain a subclone of the v-*sis* gene which had the following characteristics: (i) flanking *Xho*I (5') and *Cla*I (3') sites to facilitate insertion of the deleted v-*sis* genes into a retroviral vector, and (ii) a unique restriction site at or near the TAA codon of the v-*sis* gene. The 1,190-base pair *Sal*I (5')-to-*Xba*I (3') fragment of SSV containing the entire *env-sis* coding region was previously subcloned into a pBR322 derivative, pDD161. The desired flanking sites were introduced by linker insertion, resulting in plasmid pDD164. A 429-base pair 5' fragment of the v-*sis* gene was removed from pDD164 by digestion with *Bgl*II and *Xho*I, followed by fill-in and ligation; this resulted in the restoration of both sites. The 429-base pair fragment which was removed contained one of the two *Mst*II sites which are present in the v-*sis* gene. The resulting plasmid, pDD165, contained only one *Mst*II site, which spanned the TAA termination codon of the v-*sis* gene. This *Mst*II site was converted to a *Bam*HI site in the following way. The plasmid was linearized with *Mst*II, the 5' overhang was removed by S1 nuclease treatment and treated with the Klenow fragment of *E. coli* DNA polymerase I in the presence of the four deoxynucleoside triphosphates, and a *Bam*HI linker (CCG GATCCGG) was ligated into the molecules. The resulting plasmid, pDD175, lacked the TAA terminator codon of the v-*sis* gene and contained a unique *Bam*HI site in place of the *Mst*II site at nucleotide 813. The 5' end of the v-*sis* gene was then inserted into pDD175 as a *Xho*I-to-*Bgl*II fragment, resulting in pMS001. This plasmid contained the entire v-*sis* gene flanked by a 5' *Xho*I site and a 3' *Cla*I site and had a unique *Bam*HI site in place of the TAA terminator codon of the v-*sis* gene. Plasmid pMS001 was linearized by digestion with *Bam*HI, and the 5' overhang was filled in by using the Klenow fragment and deoxynucleoside triphosphates. *Bal* 31 digestions were carried out according to the instructions of the suppliers by using a *Bal* 31 concentration of 0.3 U/μg of DNA. Digestions were carried out at 30°C for time periods ranging from 20 s to 2 min. *Bal* 31 digestion was stopped by phenol extraction and ethanol precipitation. Following treatment of the molecules with the Klenow fragment, a self-complementary 26-mer oligonucleotide, TCAATCAG TCAAGCTTGACTGAT TGA, was ligated into the molecules. The molecules were screened for the presence of the *Hin*dIII site present in the termination oligonucleotide and for the presence of the *Cla*I site at the 3' end of the v-*sis* insertion. A total of 17 deletion mutants of the v-*sis* gene were constructed in this fashion. The extent of each deletion was determined by nucleotide sequencing into the v-*sis* gene from the *Hin*dIII site, using the method of Maxam and Gilbert (18).

In order to allow expression of the deleted v-*sis* genes in NIH 3T3 cells, the deletions of the v-*sis* gene were inserted into pDD102, a Moloney murine leukemia virus (M-MuLV)-derived expression vector (1a, 9). The subclones containing the deleted v-*sis* genes consisted of two classes. One class contained the v-*sis* gene as an *Xho*I-to-*Xho*I insertion, while the other contained the v-*sis* gene as an *Xho*I-to-*Cla*I insertion. The v-*sis* genes which were flanked by *Xho*I sites were inserted into pDD102 as *Xho*I-to-*Xho*I fragments. We constructed a variant of pDD102, pMS020, in which a *Cla*I site was inserted immediately after the *Xho*I site. This allowed insertion of the v-*sis* genes which were flanked by *Xho*I and *Cla*I sites. The advantage of the latter method is that the correct orientation of the insertion was achieved in more than 95% of the cases, while with the other method at best 50% of the recombinant clones contained the v-*sis* insertion in the correct orientation for expression. In all cases, the correct orientation of the inserted v-*sis* gene was confirmed by restriction mapping of the plasmid. In order to allow expression of the v-*sis* genes in COS-1 cells, the v-*sis* genes

were inserted into the unique *Xho*I site of pJC119 (26). This resulted in the v-*sis* genes being put under the transcriptional control of the simian virus 40 late promoter.

Focus assays and virus titers. Focus assays and viral titrations were performed essentially as described previously (1, 8). Briefly, semiconfluent monolayers of NIH 3T3 cells grown in 60-mm dishes were transfected with a calcium phosphate precipitate containing 1 μg of a v-*sis* DNA clone, 1 μg of a DNA clone of replication-competent M-MuLV (12), and 20 μg of sheared calf thymus DNA. A calcium phosphate precipitate was formed by adding 400 μl of 0.25 M $CaCl_2$ to the DNA in 400 μl of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–280 mM NaCl–10 mM sodium phosphate (pH 7.0°). The solution was allowed to stand for 45 min before it was added directly to the medium containing cells. The precipitate was left on the cells overnight before we fed the preparations and split them into dishes (4 by 10 cm) the next day. The cells were fed at 3-day intervals and scored at 7 to 10 days posttransfection. The conditioned medium was collected when the assays were scored and titrated onto fresh monolayers of NIH 3T3 cells for the presence of focus-forming virus. Typically, 200 μl of an appropriate dilution of conditioned medium containing 4 μg of Polybrene per ml was allowed to adsorb to the cells for 1 h. The cells were washed, fed, and scored after 4 to 5 days. All cells were grown in Dulbecco modified minimal essential medium supplemented with 10% calf serum.

Immunoprecipitation. Semiconfluent monolayers of COS-1 cells grown in 60-mm dishes were transfected with DNAs by using the DEAE-dextran method. The cells were washed twice with Tris-saline (10 mM Tris, pH 7.4, 150 mM NaCl), a solution of Tris-saline containing 5 μg of DNA per ml and 50 μg of DEAE-dextran (molecular weight, 2.0×10^6) per ml was added to the cells, and the preparation was incubated at 37°C for 10 min. The DNA solution was removed, and the cells were shocked with a solution of 10% dimethyl sulfoxide in Tris-saline at room temperature for 1 min. The cells were washed twice with Tris-saline and fed with fresh medium containing 100 μM chloroquine for 2 h. The cells were then fed with fresh medium for 48 h before they were labeled. The cells were labeled with 100 μCi of [^{35}S]cysteine and 100 μCi of [^{35}S]methionine in Dulbecco modified Eagle medium lacking cysteine and methionine for 2 h. For immunoprecipitation of intracellular proteins, cell lysates were prepared in RIPA buffer (10 mM sodium phosphate, pH 7.0, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1% Trasylol), and cellular debris was pelleted before the sample was divided for immunoprecipitation. Immunoprecipitation was performed as described previously (13) by using *Staphylococcus* protein A to collect the immunoglobulin G. The immunoprecipitates were suspended in sample buffer (50 mM Tris, pH 6.8, 2% SDS, 20% β-mercaptoethanol, 10% glycerol), boiled for 2 min, and clarified before electrophoresis on 15% SDS–polyacrylamide gels. For the samples that were analyzed under nonreducing conditions, the immunoprecipitates were suspended in sample buffer lacking β-mercaptoethanol. For the immunoprecipitates that were treated with endoglycosidase F, the immunoprecipitates were suspended in Endo F reaction buffer (5) and boiled for 2 min. The *Staphylococcus* protein A was pelleted, and 1-μl portions of Endo F were added to the samples before overnight incubation at 25°C. Then 2× sample buffer was added, and the samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). For immunoprecipitation of conditioned medium, the conditioned medium was collected, lyophilized, and suspended in 200 μl of RIPA buffer before immunoprecipitation.

RESULTS

The predicted amino acid sequence of the v-*sis* gene product is homologous to the amino acid sequence of PDGF from residue 112 to 220 (15, 16, 29), with the result that the v-*sis* gene product contains 51 more residues at its C terminus than PDGF does. In order to understand how much of the C terminus of the wild-type v-*sis* (v-*sis*^{wt}) gene product is dispensable for transformation, we constructed a series of mutants with deletions in the v-*sis* gene (Fig. 1).

Two methods were used to ensure that the resulting deletions of the v-*sis* gene encoded proteins with defined C termini. In one approach, the out-of-frame TGA codon located at nucleotides 712 to 714 was inserted into the correct reading frame to provide for translation termination. When this method was used, deleted v-*sis* genes that were expected to encode truncated gene products 239, 223, 177, and 136 amino acids long were constructed. An additional mutant with an internal deletion of the v-*sis* gene in which the correct reading frame was maintained was also constructed. This v-*sis* deletion resulted in the removal of residues 178 to 238 from the v-*sis*^{wt} gene product.

Additional deletion mutants with mutations in the v-*sis* gene were constructed by *Bal* 31 mutagenesis. The v-*sis* gene was then linearized at its 3' end and treated with *Bal* 31 exonuclease for varying amounts of time. The DNA ends were treated with the Klenow fragment of *E. coli* DNA polymerase I, and this was followed by ligation of a termination oligonucleotide into the molecules. This oligonucleotide, TCAATCAGTCAAGCTTGACTGATTGA, contained TGA codons in all possible reading frames, thereby ensuring that the deleted v-*sis* genes encoded truncated gene products with defined termini. The exact positions of the deletions were determined by nucleotide sequencing, and the predicted amino acid sequences were deduced for these mutants (Table 1). Using this method, we constructed an additional 17 C-terminal deletion mutants of the v-*sis* gene that encoded truncated gene products which were 121 to 246 amino acids long.

The deletion mutants with mutations in the v-*sis* gene were assayed for their ability to induce focus formation in NIH 3T3 cells. For these experiments we used a M-MuLV-derived expression vector which provided for the expression of inserted genes by using the M-MuLV *env* transcription pathway (1a, 10). The v-*sis* genes were inserted into the retroviral vector, introduced into NIH 3T3 cells by the calcium phosphate coprecipitation method, and assayed for their ability to induce the formation of foci (Fig. 1). Since the expression vector was defective in both the *gag* and *pol* genes, a replication-competent DNA clone of M-MuLV (12) was cotransfected in order to provide for viral spread and rescue. Biological activities of up to 5.9×10^4 foci per pmol were obtained from transfection experiments with the v-*sis*^{wt} gene (Table 1). Mutants in which up to 57 residues were deleted from the C terminus of the v-*sis*^{wt} gene product (for example, v-*sis*^{214}) possessed biological activity equal to that of the v-*sis*^{wt} gene. However, v-*sis*^{204}, which encoded a protein lacking the 67 C-terminal residues, was inactive when it was assayed for focus formation. Other mutant v-*sis* genes that encoded proteins in which up to 150 C-terminal or 62 internal amino acids had been removed were also biologically inactive. The conditioned media from the focus assays were collected and titrated onto fresh monolayers of NIH

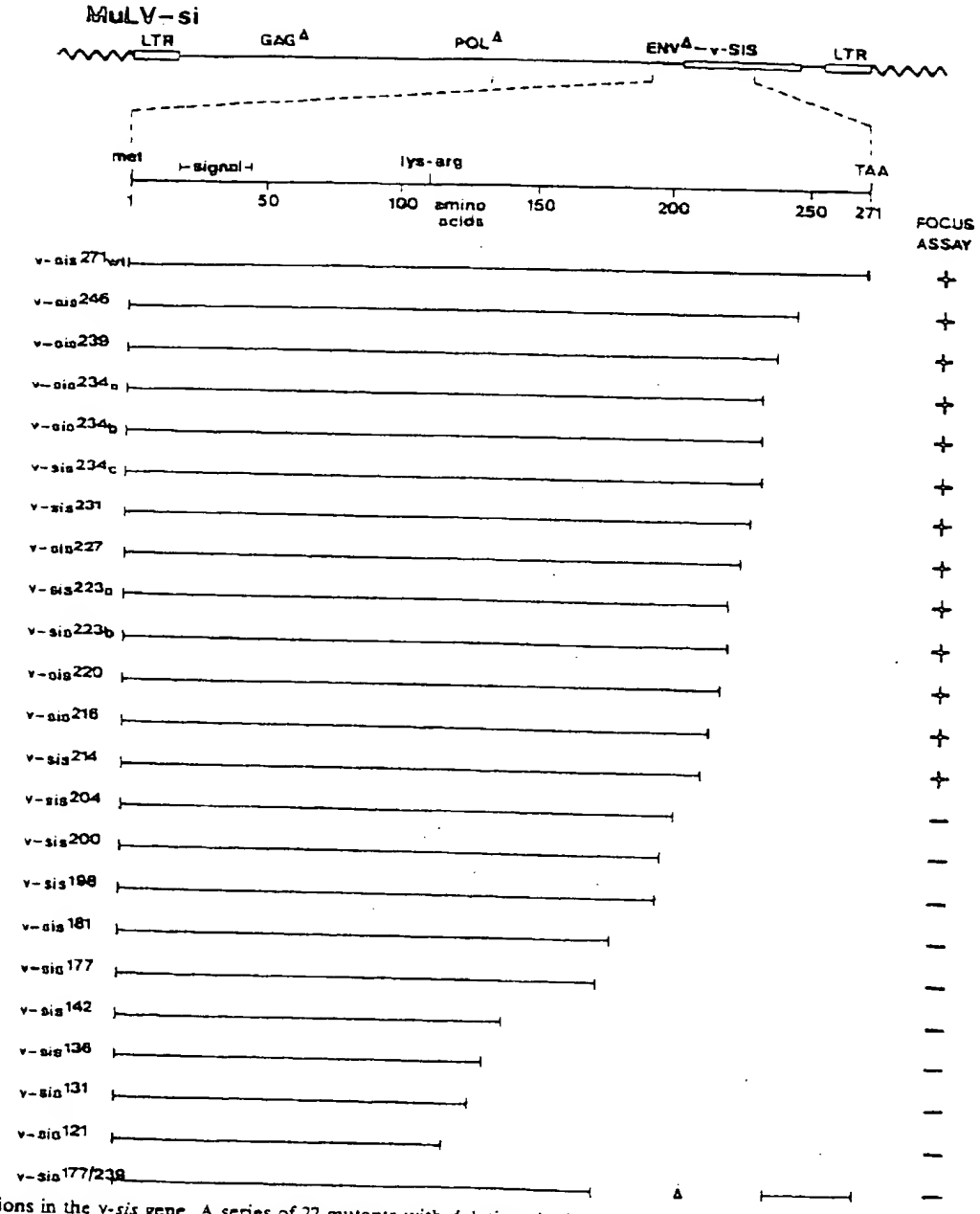

FIG. 1. Deletions in the v-*sis* gene. A series of 22 mutants with deletions in the v-*sis* gene were constructed as described in the text. The endpoint of each deletion was determined by nucleotide sequencing, and the superscript of each deletion designation indicates the number of amino acids encoded by the deleted v-*sis* gene. The number of amino acids was determined by counting from the N terminus of the predicted initial translation product of the v-*sis* gene. As shown in Table 1, the deletions had slightly different termini, depending on the exact reading frame into the termination oligonucleotide. For example, three independent isolates of the v-*sis*^{234} gene were isolated, each possessing a different C terminus; these were designated v-*sis*^{234}a, v-*sis*^{234}b, and v-*sis*^{234}c. A map of the v-*sis*^{mt} gene is shown at the top. The v-*sis* deletions were inserted into the M-MuLV-derived expression vector shown at the top. The plus and minus signs indicate the biological activities of the individual v-*sis* deletions as determined by their ability to induce the formation of foci in NIH 3T3 cells and by the titer of rescuable focus-forming virus. The values obtained in these experiments are shown in Table 1. LTR, Long terminal repeat.

3T3 cells in order to assay for the presence of rescuable focus-forming virus. The transforming v-*sis* genes had high levels of rescuable focus-forming virus in the conditioned media from the focus assays, while the v-*sis* genes which gave no detectable foci in the focus assays also lacked detectable focus-forming virus in the conditioned media. The sharp difference in biological activity between the v-*sis*^{214} and v-*sis*^{204} genes allowed the C-terminal limit of the biolog-

TABLE 1. Biological activities of the deletion mutants of the v-*sis* gene

Deletion mutant	Plasmid[a]	Exact endpoint of mutant		No. of residues deleted[d]	Results of biological assays	
		Nucleotide[b]	C-terminal sequence class[c]		Focus assay (FFU/pmol)[e]	Titer (FFU/ml)[f]
v-*sis*^{271}	pDD145	813	wt	0	5.9×10^{4}	2.5×10^{6}
v-*sis*^{246}	pMS042	737	C	25	4.8×10^{3}	3.0×10^{5}
v-*sis*^{239}	pMH75	716	D	32	4.4×10^{3}	8.0×10^{5}
v-*sis*^{234}a	pMS030	704	C	37	3.4×10^{4}	1.0×10^{7}
v-*sis*^{234}b	pMS036	702	A	37	1.9×10^{4}	6.0×10^{6}
v-*sis*^{234}c	pMS034	701	C	37	1.4×10^{4}	1.0×10^{7}
v-*sis*^{231}	pMS027	693	A	40	3.5×10^{4}	5.0×10^{5}
v-*sis*^{227}	pMS040	681	A	44	9.6×10^{3}	1.7×10^{6}
v-*sis*^{223}a	pMS034	668	C	48	3.4×10^{4}	5.0×10^{6}
v-*sis*^{223}b	pMH67	668	D	48	2.4×10^{4}	1.5×10^{5}
v-*sis*^{220}	pMS044	660	A	51	2.9×10^{4}	2.0×10^{6}
v-*sis*^{216}	pMS029	647	C	55	5.8×10^{4}	1.0×10^{7}
v-*sis*^{214}	pMS033	642	A	57	4.3×10^{4}	3.7×10^{6}
v-*sis*^{204}	pMS052	613	B	67	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*^{200}	pMS039	599	C	71	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*^{198}	pMS037	594	A	73	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*^{181}	pMS038	544	B	90	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*^{177}	pMH76	531	E	94	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*^{142}	pMS028	427	B	129	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*^{136}	pMH68	408	F	135	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*^{131}	pMS032	394	B	140	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*^{121}	pMS041	362	C	150	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$
v-*sis*$^{177/239}$	pMH66	813	wt	62	$<1.0 \times 10^{1}$	$<5.0 \times 10^{0}$

[a] Plasmids which contained the deletion mutants in the M-MuLV-derived expression vector.
[b] Numbers of the last nucleotides derived from the v-*sis* gene, counting from the first ATG codon of the *env-sis*-open reading frame; these numbers can be converted to the nucleotide numbers of the SSV sequence (3) by adding 3,657.
[c] C-terminal amino acid sequences of the deletion mutants. The letters indicate in which class the C-terminal amino acid sequence of a deletion mutant belongs, depending on the reading frame into the termination oligonucleotide. The classes were defined as follows: class A deletion mutants contained the amino acid sequence Ser-Ile-Ser-Gln-Ala at their C termini; class B mutants contained the sequence X-Asn-Gln-Ser-Ser-Leu-Thr-Asp, where X was Leu (except for the v-*sis*^{131} gene product, which had a Val at this position); class C mutants had the amino acid sequence Z-Gln-Ser-Val-Lys-Leu-Asp at their C termini, where Z was the amino acid sequence of the v-*sis*^{wt} gene product at this position (except for the v-*sis*^{234} c gene product, which had a His in place of the Gln found in the v-*sis*^{wt} gene product); class D mutants had the amino acid sequence of the v-*sis*^{wt} gene product at the position indicated; class E mutants ended in Val-Thr; and class F mutants ended in Leu. wt, Wild type.
[d] Numbers of amino acids which were deleted from the v-*sis*^{wt} gene product.
[e] FFU, Focus-forming units.
[f] Titers of rescuable focus-forming virus present in the conditioned media of the focus assays.

ically active v-*sis* gene product to be placed between residues 214 and 204.

In order to obtain high levels of the gene products coded for by the deleted v-*sis* genes, we used a transient expression assay. Transient expression in COS-1 cells is a widely used method which allows high levels of expression of a desired gene (7, 23, 26, 28). Furthermore, the ability of COS-1 cells to proteolytically process and secrete hormones in a biologically active form has been well established (28). For these experiments we used simian virus 40-derived expression vector pJC119, which allowed for the expression of genes inserted at a unique *Xho*I site by using the late transcription pathway (26). Two mutant v-*sis* genes which encoded transforming gene products were inserted into pJC119 in the correct orientation for expression and then introduced into COS-1 cells. Cell lysates prepared from metabolically labeled cells were subjected to immunoprecipitation with antiserum directed against a peptide corresponding to residues 52 to 61 of the predicted v-*sis*^{wt} gene product. Cells transfected with the v-*sis*^{wt} gene yielded a 32-kDa protein when they were analyzed under reducing SDS-PAGE conditions (Fig. 2a, lane 1), while cells transfected with the v-*sis*^{223} or v-*sis*^{239} gene contained 26- and 28-kDa proteins (Fig. 2a, lanes 3 and 5). When the immunoprecipitates were analyzed under nonreducing conditions, the transforming gene products dimerized, yielding proteins of the expected sizes (Fig. 2b, lanes 1, 3, and 5).

The v-*sis*^{wt} gene product is proteolytically processed at a basic dipeptide, Lys-Arg at residues 110 and 111, resulting in the formation of a PDGF-related 20-kDa protein (21). In order to determine whether the truncated v-*sis* gene products encoded by the mutant genes are proteolytically processed, cell lysates from COS-1 cells transfected with the v-*sis*^{239} and v-*sis*^{223} genes were immunoprecipitated w[illegible] anti-PDGF serum. We found that the v-*sis*^{239} and v-*sis*[illegible] genes encoded PDGF-related protein doublets (16.5 and 16 kDa and 14.5 and 14 kDa, respectively) in addition to the 28- and 26-kDa proteins also detected with the N-terminal peptide serum (Fig. 3, lanes 1 and 3). When the immunoprecipitates were analyzed under nonreducing conditions, PDGF-related dimers of the appropriate sizes were found (Fig. 3, lanes 5 and 7). The sizes of the smaller PDGF-related proteins are consistent with proteolytic processing at the Lys-Arg dipeptide. No smaller PDGF-related proteins were specifically immunoprecipitated from COS-1 cells that expressed the v-*sis* genes.

The conditioned medium of the transfected COS-1 cells was also examined for the presence of PDGF-related proteins. We found that PDGF-related 16.5- and 16-kDa proteins and 14.5- and 14-kDa proteins were secreted into the media from COS-1 cells transfected with the v-*sis*^{239} and v-*sis*^{223} genes, respectively (Fig. 3, lanes 4 and 2). These proteins dimerized when they were analyzed under nonreducing conditions (Fig. 3, lanes 6 and 8) and comigrated with the intracellular processed forms of the mutant v-*sis* gene products, indicating that no further proce[illegible]

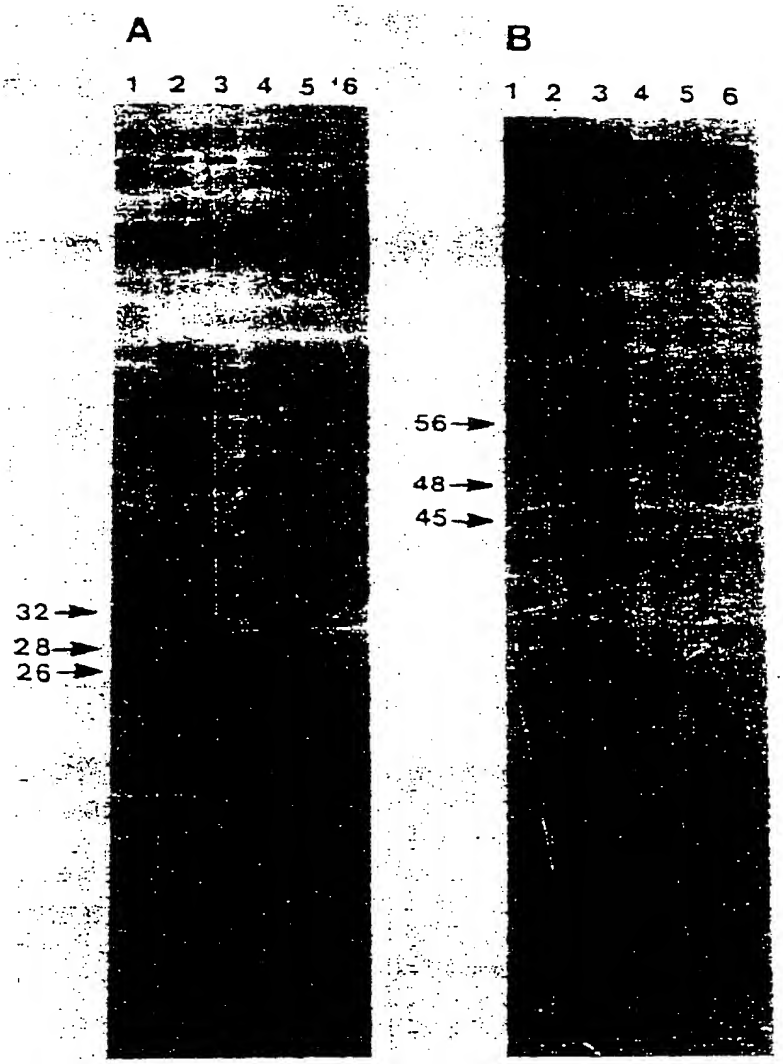

FIG. 2. Expression of the transforming v-*sis* genes in COS-1 cells. The v-sis^{p1} gene (lanes 1 and 2), the v-sis^{222} gene (lanes 3 and 4), and the v-sis^{239} gene (lanes 5 and 6) were expressed in COS-1 cells under the transcriptional control of the simian virus 40 late promoter. Lysates from cells metabolically labeled with [^{35}S]cysteine were immunoprecipitated with antiserum raised against a peptide corresponding to residues 52 through 61 of the predicted amino acid sequence of the v-*sis* gene product. (A) Samples analyzed under reducing conditions. (B) Identical samples analyzed under nonreducing conditions. Lanes 1, 3, and 5 contained immunoprecipitates with the antipeptide serum, while in lanes 2, 4, and 6 the antipeptide serum was preincubated with an excess of the peptide before immunoprecipitation. The arrows indicate the positions of 32-, 26-, and 28-kDa proteins in panel A and the positions of the dimer forms of these proteins in panel B.

occurred during secretion. Further evidence that the v-*sis* gene products are secreted from COS-1 cells was obtained by testing the conditioned media of transfected COS-1 cells for the presence of mitogenic activity. Cells transfected with the transforming v-*sis* genes secreted a mitogenic activity into the media, while cells transfected with either nontransforming v-*sis* genes or vector DNA alone contained no mitogenic activity in their conditioned media (data not shown).

We also expressed the v-sis^{214} and the v-sis^{204} genes in COS-1 cells, since these genes defined the limit of the C terminus of the v-*sis* gene product which is required for transformation. When COS-1 cell lysates were immunoprecipitated with antiserum raised against the bacterially expressed gene product and analyzed under reducing SDS-PAGE conditions, we found that the v-sis^{214} and v-sis^{204} genes encoded 25- and 24-kDa proteins (Fig. 4a, lanes 3 and 4). The 1-kDa difference between the proteins is consistent with the eight-amino acid difference between the two mutant v-*sis* genes. However, only the v-sis^{214} gene encoded a protein that was capable of dimerization when preparations were analyzed under nonreducing SDS-PAGE conditions (Fig. 4b, lanes 3 and 4). Smaller PDGF-related 13.5- and 13-kDa proteins were detected in lysates of cells transfected with the v-sis^{214}gene, but they were not detected in lysates from cells transfected with the v-sis^{204} gene, indicating that only the v-sis^{214} gene product was proteolytically processed at the Lys-Arg dipeptide. Several other

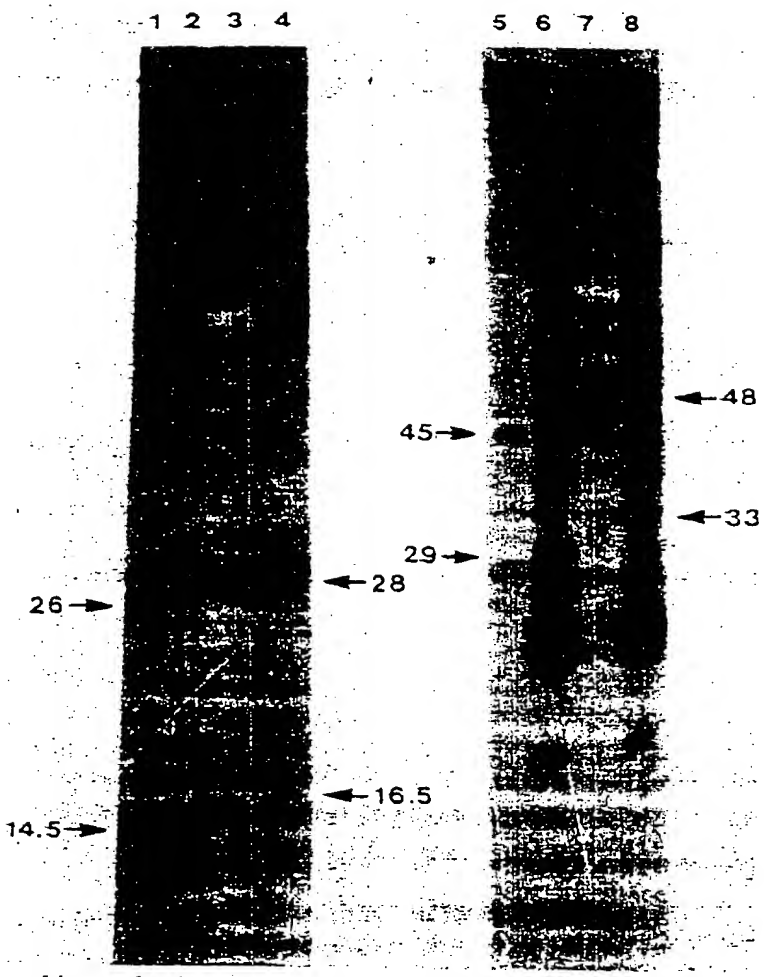

FIG. 3. Immunoprecipitation of the transforming v-*sis* gene products with anti-PDGF serum. COS-1 cells were transfected with the v-*sis*^{223} gene (lanes 1, 2, 5, and 6) and the v-*sis*^{239} gene (lanes 3, 4, 7, and 8) and metabolically labeled with [^{35}S]cysteine. Lysates (lanes 1, 3, 5, and 7) and conditioned media (lanes 2, 4, 6, and 8) were immunoprecipitated with anti-PDGF serum and analyzed under reducing (lanes 1 through 4) or nonreducing (lanes 5 through 8) SDS-PAGE conditions. The arrows indicate the positions of full-length PDGF-related 26- and 28-kD proteins in lanes 1 through 4. The positions of the doublets of PDGF-related proteins (14.5 and 14 kDa and 16.5 and 16 kDa) resulting from proteolytic processing are also indicated. The positions of the dimers are indicated in lanes 5 through 8.

nontransforming mutants of the v-*sis* gene were analyzed in a similar manner, with the result that none of the nontransforming genes was found to encode a gene product which was capable of dimerization under nonreducing conditions (data not shown).

The v-*sis* gene product is modified by the addition of a high-mannose oligosaccharide to Asn residue 93 during its translocation across the membrane of the rough endoplasmic reticulum (22; submitted for publication). Although the deletion mutants retained the signal sequence encoded by the v-*sis* gene and could be localized to the Golgi apparatus by using indirect immunofluorescence (data not shown), we wanted to determine whether the mutant v-*sis* gene products were modified by glycosylation. Therefore, immunoprecipitates of several transforming and nontransforming v-*sis* gene products were treated with endoglycosidase F and analyzed by SDS-PAGE. The mobilities of both the transforming and nontransforming v-*sis* gene products were altered in a manner consistent with the presence of an N-linked oligosaccharide (Fig. 5). As in the case of the v-*sis*^{mt} gene product, the oligosaccharide added to the mutant v-*sis* gene products was of the high-mannose type, as indicated by its sensitivity to endoglycosidase H (data not shown).

DISCUSSION

We constructed a series of mutants with deletions in the v-*sis* gene which defined the C terminus of the biologically active v-*sis* gene product. Deleted v-*sis* genes which encode truncated proteins that are 214 amino acids long or longer are fully active when they are assayed for focus formation in NIH 3T3 cells. However, the removal of only 10 more residues from the C terminus of the v-*sis* gene product results in the loss of biological activity, suggesting that these residues possess an important feature for transformation.

The principal difference between the gene products encoded by the transforming and nontransforming mutants

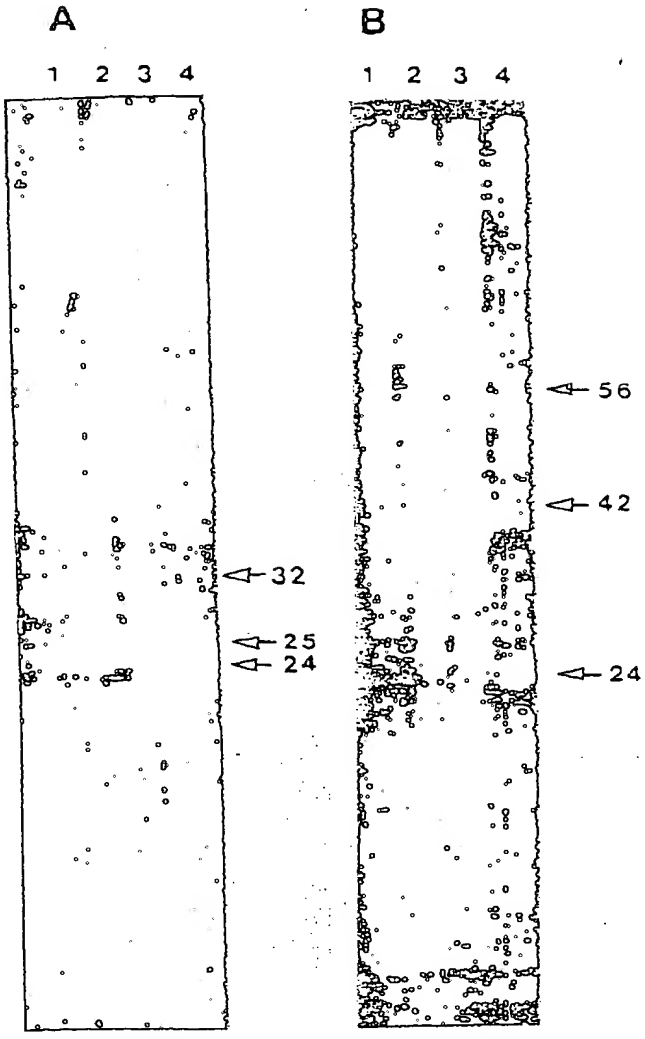

FIG. 4. Immunoprecipitation of the v-sis^{214} and v-sis^{204} gene products. COS-1 cells were transfected with vector DNA alone (lane 1), with the v-sis^{wt} gene (lane 2), with the v-sis^{214} gene (lane 3), or with the v-sis^{204} gene (lane 4). Lysates were labeled with [^{35}S]cysteine and [^{35}S]methionine and immunoprecipitated with antiserum raised against the bacterially synthesized v-*sis* gene product, and the immunoprecipitates were analyzed under reducing (A) or nonreducing (B) SDS-PAGE conditions. The arrows indicate the positions of the PDGF-related 32-, 25-, and 24-kDa proteins in panel A and the respective dimers in panel B.

the v-*sis* gene is the failure of the nontransforming gene products to dimerize. In most other respects, including translocation across the rough endoplasmic reticulum and glycosylation, the nontransforming v-*sis* gene products are indistinguishable from the transforming v-*sis* gene products. An additional difference between the transforming and nontransforming gene products is the lack of proteolytic processing of the nontransforming gene products. However, a mutant of the v-sis^{wt} gene which lacks the basic dipeptide at residues 110 and 111 retains full biological activity (submitted for publication), indicating that the lack of proteolytic processing by the nontransforming deletion mutants reported here does not explain their lack of biological activity. Our results strongly suggest that the loss of biological activity is solely due to the failure to dimerize. This conclusion is supported by the previous demonstration that both PDGF and the v-sis^{wt} gene product lose their mitogenic activity after reduction (20).

The v-*sis* gene product contains a total of 10 cysteine residues. Of these 10, cysteine residue 28 is encoded by the *env*-derived sequences of the v-*sis* gene. This residue is removed during signal sequence cleavage and probably does not contribute to the dimerization of the v-*sis* gene product. The next eight cysteine residues are located within the PDGF-homologous portion of the v-*sis* gene product, extending from residue 112 to residue 220. The tenth cysteine residue is located outside this region at residue 256. Our results indicate that this C-terminal cysteine residue is not required for dimerization, since all of the mutant v-*sis* gene products which have C termini between residue 246 and 214 are capable of forming dimers under nonreducing conditions. These mutant v-*sis* gene products are also fully active in transformation. However, cysteine residues 208 and 210, which are the eighth and ninth cysteine residues (counting from the N terminus of the predicted full-length gene product), are located within the 10 residues which separate the transforming v-sis^{214} gene product from the nontransforming v-sis^{204} gene product as the sequence Cys-Lys-Cys (Fig. 6). The failure of the v-sis^{204} gene product to dimerize suggests that one or both of these cysteine residues are involved in the formation of interchain disulfide bonds. The mere presence of a cysteine residue in the vicinity of the C terminus of the v-*sis* gene product is not sufficient for dimerization, since the v-$sis^{177/239}$ gene product, which contains an internal deletion of 62 amino acids, also fails to dimerize. This gene product contains all of the cysteine residues found in the v-sis^{wt} gene product except cysteine residues 208 and 210. These cysteine residues clearly are crucial for the formation of proper disulfide bonds in the v-*sis* gene product. Several questions regarding the importance of these cysteine residues for proper folding remain. Are both cysteine residues 208 and 210 involved in the formation of interchain disulfide bonds, and are both required for dimerization, or is one sufficient? With which other cysteine residues do these residues form disulfide bonds? What is the importance of local secondary structure for the formation and stability of the proper disulfide bonds? Site-directed mutagenesis of these cysteine residues should allow these and other questions to be answered.

The v-*sis* gene product is proteolytically processed at a Lys-Arg dipeptide (residues 110 and 111), resulting in the formation of a PDGF-related 20-kDa protein (21). This protein contains 8 of the 10 cysteine residues and is capable of dimerization under nonreducing conditions. We found that the gene products encoded by the deletion mutants of the v-*sis* gene are also proteolytically processed at this dipeptide. It is interesting that only the transforming gene products are proteolytically processed at this dibasic residue. Although processing at this dipeptide is not required for transformation, the smaller PDGF-related forms of the v-*sis* gene product resulting from this processing event are presumably biologically active in transformation, since they retain the mitogenic activity of the v-*sis* gene product. This allows definition of the minimal transforming region of the v-*sis* gene, which consists of 103 amino acids extending from residue 112 to residue 214 (Fig. 6). A total of six residues can be removed from the C terminus of the PDGF-homologous

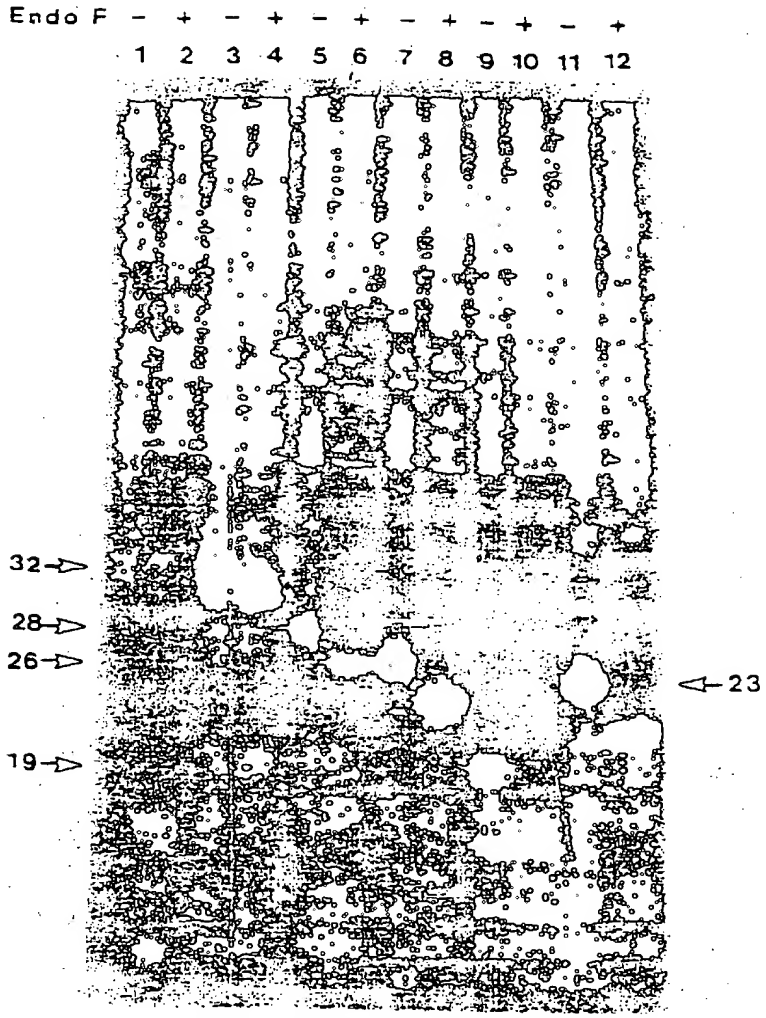

FIG. 5. Glycosylation of the mutant v-*sis* gene products. COS-1 cells were transfected with vector DNA alone (lanes 1 and 2), the v-*sis*[214] gene (lanes 3 and 4), the v-*sis*[239] gene (lanes 5 and 6), the v-*sis*[223] gene (lanes 7 and 8), the v-*sis*[177] gene (lanes 9 and 10), or the v-*sis*[177/239] gene (lanes 11 and 12). Lysates were labeled with [^{35}S]cysteine and [^{35}S]methionine, immunoprecipitated with antipeptide serum, and not treated (lanes 1, 3, 5, 7, 9, and 11) or treated (2, 4, 6, 8, 10, and 12) with endoglycosidase F. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions. The arrows indicate the positions of the glycosylated forms of the v-*sis*-encoded 28-, 26-, 19-, and 23-kDa proteins which are present in lanes 3, 5, 7, 9, and 11.

region of the v-*sis* gene product with no loss of biological activity.

The v-*sis* gene product has been reported to undergo proteolytic processing at its C terminus, resulting in the formation of a PDGF-related protein which is detected under nonreducing conditions as a 24-kDa dimer (21). The site of C-terminal processing and its importance for transformation are not known. Furthermore, the nature of the subunits of this dimer is not known, since they have not been detected under reducing conditions. The size of the fully processed v-*sis* gene product, which presumably is composed of two 12-kDa subunits, is approximately the same size as the gene product encoded by the minimal transforming region defined by the v-*sis*[214] gene. If this is the case, it suggests that cysteine residues 208 and 210 are retained after C-terminal processing. This would agree with the detection of this form of the v-*sis* gene product under nonreducing conditions. However, the failure to detect the subunits which compose the 24-kDa dimer under reducing conditions raises the possibility that this protein is not derived from C-terminal processing of the v-*sis* gene product, but is a result of some other type of modification or is unrelated to the v-*sis* gene product altogether. The nature of the proposed proteolytic event which results in the formation of the 24-kDa dimer is also not known. There are no basic dipeptide residues in the vicinity of cysteine residues 208 and 210 that could be a possible site(s) of proteolytic processing. Our failure to detect a C-terminal processed v-*sis* gene product in COS-1 cells is further evidence that this processing event does not occur at basic dipeptide residues, since COS-1 cells do possess the enzymes required for proteolysis at basic dipeptides (28). However, it is possible that the C-terminal p

FIG. 6. Minimal transforming region of the v-*sis* gene product. The entire amino acid sequence of the v-*sis* gene product is given by using the single-letter code. The signal sequence residues of the v-*sis* gene product are also indicated with italics. The minimal transforming region of the v-*sis* gene product, defined in this study as amino acid residues 112 to 214, is shown in large letters; the rest of the sequence is shown in small letters. The cysteine residues are indicated by asterisks. The C terminus of chain B of PDGF from amino acid sequence data (15) is indicated by the arrow above residue 220. The single-letter amino acid code used is: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

essing of the v-*sis* gene product represents a novel proteolytic event. The nature and importance of the putative C-terminal processed form of the v-*sis* gene product will require further investigation.

The C-terminal amino acid sequences of some of the mutant v-*sis* gene produts are different from the predicted amino acid sequence of the v-*sis*^{m1} gene product as a result of different reading frames in the termination oligonucleotide (Table 1). However, two lines of evidence indicate that these extra C-terminal amino acids have little or no effect on the biological activity of the deleted v-*sis* genes. First, some deletions in the v-*sis* gene which retained the same number of v-*sis*-encoded amino acids but utilized different TGA codons of the linker were found to have the same biological activity. Second, the biological activity of the v-*sis* deletions followed a clear pattern which correlated only with the extent of the deletion itself.

A secreted form of the v-*sis* gene product has been identified in conditioned media of SSV-transformed cells as a 20-kDa protein (6) or a 17-kDa protein (20). The transforming v-*sis* deletion mutants described here also secreted a PDGF-related protein into the media. The secreted form most likely results from N-terminal processing at residues 110 and 111, but is not processed at the C terminus. The secretion of a PDGF-related protein from the transforming v-*sis* deletions is supported by our finding of a mitogenic activity in the conditioned media of COS-1 cells that express the transforming v-*sis* genes.

The best correlation that we have found between the biological properties of the v-*sis* deletion mutants and the physical properties of the encoded proteins is between transformation and dimerization. The connection between dimerization and transformation has recently been described on the basis of two mutants with mutations in the v-*sis* gene which encode truncated gene products that differ in size by 50 amino acids (17). Our data support previous work with both PDGF and the v-*sis* gene product, which showed that mitogenic activity was lost upon reduction (20). These results strengthen the relationship between the two biological properties of the v-*sis* gene product, transformation and mitogenesis. However, why the mitogenic activity of the v-*sis* gene product results in transformation while the mitogenic activity of PDGF does not is not known. Both the v-*sis* gene product and PDGF are able to stimulate autophosphorylation of the cell surface PDGF receptor (13, 19, 20, 25). However, it has recently been proposed that the v-*sis* gene product is able to interact with the PDGF receptor inside the cell, perhaps during transport through the Golgi

apparatus and exocytotic vesicles (11, 14). Alternatively, it is possible that the v-*sis* gene product, since it lacks subunit A of PDGF, has a different effect on the PDGF receptor than PDGF does. In either case, it is clear from our results that dimerization of the v-*sis* gene product as a B-B homodimer is required for transformation.

ACKNOWLEDGMENTS

We thank Tom Deuel for his gift of anti-PDGF serum, Gernot Walter and Frederick Graesser for their gift of the antipeptide serum used in this work, and Jay Sweet for his gift of antisera raised against the bacterially synthesized v-*sis* gene product. We also thank Rick Bold and Karen May for excellent technical assistance.

This work was supported by Public Health Service grant CA40573 from the National Cancer Institute. M.H. was supported by Public Health Service Cell and Molecular Biology Training Grant GM07313 from the National Institutes of Health. M.K.S. was supported by a postgraduate scholarship from the Natural Sciences and Engineering Research Council of Canada. D.J.D. gratefully acknowledges receipt of support from the Chicago Community Trust/Searle Scholars Program.

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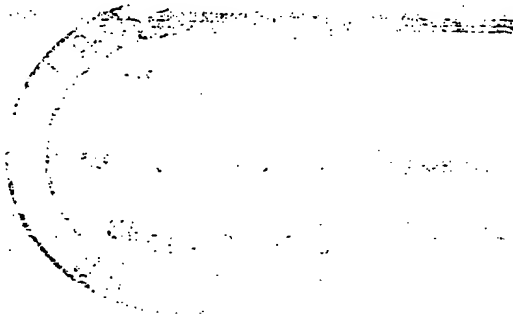
Molecular and Cellular Biology



Published monthly by the American Society for Microbiology

DEN: MCEBD4 6 (4) 969–1362 (1986)

ISSN: 0270-7306

Express Mail No.:_____

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Hu *et al.*

Application No.: 08/107,997 | Group Art Unit: 1647

Filed: June 30, 1998 | Examiner: C. Saoud

For: Vascular Endothelial Growth Factor 2 | Attorney Docket No.: PF112P4

DECLARATION OF STUART AARONSON
UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, STUART AARONSON, do declare that:

1. I am currently the Director of the Derald H. Ruttenberg Cancer Center for the Mount Sinai Medical Center in New York, New York. Since the 1970s, my research has focused on growth factors and their role in tumorigenesis and cancer as evidenced by my curriculum vitae, attached hereto as Exhibit A, which lists the publications that I have authored or co-authored. My research in the area of the molecular biology of growth factors and their receptors, including keratinocyte growth factor, fibroblast growth factor and vascular endothelial growth factor, has encompassed mammalian models of tumorigenesis, including human tumor model systems.

2. I have been asked by the Patent Attorneys representing Human Genome Sciences ("HGS") to review the specification to U.S. application serial no. 08/207,550, filed March 8, 1994, ("the HGS patent specification") and the specification to application serial no. 08/107,997, filed June 30, 1998. I have

also been asked to review and comment on the experimental evidence provided in Dr. Susan Power's Statutory Declaration, attached hereto as Exhibit B, which has been submitted in connection with proceedings related to Australian Patent Application Au-B-696764 (73941/94) in the name of HGS, entitled "Vascular Endothelial Growth Factor-2." I have also been asked to provide my comments and opinions as to what the patent specification would provide to one familiar with the molecular biology of growth factors, *e.g.*, a post doctorate or Ph.D. candidate in a research laboratory, as of the earliest filing date of the HGS patent specification, March 1994. For purposes of this analysis, I considered not only what I knew and appreciated at the relevant time, but what was expected to be known by graduate students and postdoctoral fellows who were in my laboratory at the relevant time.

3. I have reviewed and analyzed the polynucleotide and amino acid sequence identified by HGS to encode the human VEGF-2 protein, as set forth in Figure 1 of the HGS patent specification. The HGS patent specification describes, but is not limited to, the characterization of the VEGF-2 sequence and encoded protein. The HGS patent specification describes the human VEGF-2 protein as structurally related to the PDGF/VEGF family, a known family of secreted growth factors. The HGS patent specification further discloses that the VEGF-2 polynucleotide is predicted to contain an open reading frame of approximately 1050 residues, which encodes VEGF-2. (*See* the HGS patent specification at page 5, lines 25-27). The specification reports that at the amino acid level, VEGF-2 exhibits the highest homology to vascular endothelial growth factor (30% identity), followed by PDGF alpha (23%) and PDGF beta (22%). (*See* the HGS patent specification at page 5, lines 28-31). The HGS patent specification further characterizes the VEGF-2 protein as containing eight cysteines which are conserved among all known members of the PDGF/VEGF family, and in addition, also contains the fourteen amino acid signature motif, PXCVXXXRCXGCCN, found in all members of the

PDGF/VEGF family. (*See* the HGS patent specification at page 5, lines 31-33).

4. Based on the characterization of the VEGF-2 protein set forth in the HGS patent specification, one would recognize that the protein was a member of the PDGF/VEGF family of growth factors. The PDGF/VEGF family of growth factors, like other growth factors, are normally secreted in order to exert their growth promoting or mitogenic effects. Since all previously identified members of the PDGF/VEGF family were known to be secreted, one would expect the newly identified VEGF-2 to also be secreted.

5. By March 1994 it was well known to me and, I believe to my colleagues in the angiogenic field that the PDGF/VEGF family of growth factors were expressed initially as precursor proteins which underwent proteolytic processing resulting in a mature, secreted form of the protein. Thus, I would have predicted that VEGF-2 would be processed in a similar way. The 350 amino acid sequence set forth in Figure 1 of the HGS patent specification contains the conserved, signature motifs for an active form of a protein belonging to the PDGF/VEGF family. Thus, in March 1994, I would have predicted that the protein encoded by the sequence disclosed in Figure 1, expressed and secreted by a host cell as taught in the HGS patent specification, would contain those signature motifs characteristic of the PDGF/VEGF family and be biologically active.

The HGS Patent Specification Provides Sufficient Information Such that a Molecular Biologist Recognizes the Possession of a Mature Biologically Active Form of VEGF-2

6. The question of whether the 350 amino acid sequence as set forth in the HGS patent specification does indeed contain sufficient information to result in the mature processed form of VEGF-2 when secreted from a host cell has, in my opinion, been addressed and affirmatively confirmed in the experiments

reported in Dr. Susan Power's Statutory Declaration (attached hereto as Exhibit B).

7. The experiment set forth in Dr. Power's Declaration describes the use of two constructs: the 350 amino acid sequence, containing the nucleotide sequence encoding the 350 amino acid sequence of VEGF-2 (as set forth in Figure 1 of the patent specification) in frame with a heterologous promoter and signal sequence; the 419 amino acid sequence containing the nucleotide sequence encoding the 419 amino acid sequence of VEGF-2 in frame with a heterologous promoter. These two constructs were used to transform a mammalian cell line. The cells were cultured under conditions to allow the cells to express the gene products encoded by the vectors. At various time points the cell lysates and culture medium were collected and each was assayed for the presence of VEGF-2. The presence of VEGF-2 was determined by a Western blot analysis using a polyclonal antibody to VEGF-2 that recognizes both the unprocessed precursor form as well as the processed, secreted form of VEGF-2 (*See* ¶ 13 of Dr. Power's Declaration).

8. The results of the experiments reported in Dr. Power's Declaration demonstrate that the construct expressing the 350 amino acid sequence of VEGF-2 contains sufficient information to allow for the natural and correct processing of the protein to a mature biologically active protein. Indeed, the secreted proteins which result from the expression of the constructs encoding the 419 amino acid sequence and the 350 amino acid sequence of VEGF-2 are indistinguishable in size. Both are secreted and processed to a mature form of the protein which resolves as a band at approximately 30 kDa, with another minor band detectable at approximately 21 kDa (*See* Figure 1 of Dr. Power's Declaration, at Gel 3, lanes 22 and 24). Since the filing of the HGS patent specification, the 30 kDa and the 21 kDa species have been consistently identified in the art as processed mature forms of VEGF-2 with biological activity. (*See*, Joukov *et al.*, 1997, EMBO J 16: 3898, at 3898, "Joukov").

9. In sum, the experimental evidence provided in Dr. Power's Declaration demonstrates that the constructs encoding the 350 amino acid sequence and the 419 amino acid sequence of VEGF-2 are both naturally, correctly and indistinguishably processed by the cell to mature forms of the protein.

The VEGF-2 Polypeptide Is Processed To Its Mature Form Similarly From Cell Type to Cell Type

10. Since the March 1994 filing date of the HGS patent specification, subsequent publications by the inventors and others have further characterized the proteolytic processing of VEGF-2. The inventors' own publications confirm that when expressed in mammalian cells, the precursor form of VEGF-2 undergoes proteolytic processing. (*See*, Hu et al., 1997, FASEB J. 11(6) :498-504, "Hu").

11. The capacity to be processed to the mature form of VEGF-2 is an inherent property of the VEGF-2 amino acid sequence. The only information necessary for processing VEGF-2 to its mature form is contained in the amino acid sequence of VEGF-2. Any host cell with proteolytic enzymes and cellular machinery for processing the VEGF-2 polypeptide, *i.e.* a mammalian cell, will naturally process VEGF-2 to its mature form. While the efficiency of the processing to the mature form of VEGF-2 can vary depending on the cell type from which VEGF-2 is expressed, the capacity to be processed to the mature form is similar from cell type to cell type. The Joukov publication compares the proteolytic processing of VEGF-2 expressed by a number of different cell lines, including COS cells, PC-3 cells, HT 1080 cells, and 293 EBNA cells. The results of this comparison demonstrate that the processing of the VEGF-2 polypeptide to its mature form is similar from cell to cell (Joukov, at page 3901, second column). Thus, while the efficiency of processing of the precursor form may vary from cell type to cell type, the capacity of the amino acid sequence of the precursor form of VEGF-2 which allows the protein to be naturally processed does not vary.

12. I note that Example 2 of the HGS specification reports the translation of a VEGF-2 polypeptide by an *in vitro* reticulocyte lysate system resulting in a protein having an estimated molecular weight of 36-38 kD (*See*, the HGS specification at page 28, lines 5-12). Given that an *in vitro* expression system was used to achieve this result, the reported molecular weight is not surprising or inconsistent as compared to the molecular weights reported for the mature form of VEGF-2 as processed by mammalian cells. *In vitro* translation systems may contain a subset of the proteolytic enzymes found in mammalian cells. Similarly, non-mammalian expression systems, such as bacteria or baculovirus host cells, also contain different proteolytic enzymes for the processing of precursor proteins to their mature form. Thus, when expression systems other than intact mammalian cells are used to express mammalian proteins, such as VEGF-2, it is not unexpected for the mature form of the protein to have a molecular weight differing from that observed in mammalian cells. In addition to differences in processing efficiencies there may also be post-translational modifications, including the attachment of different sugar residues to the protein, any of which may result in a variance in molecular weight as observed by SDS-PAGE. However, all of the information required to achieve the mature processed form of the protein lies in the amino acid sequence of the precursor protein. Thus, other than providing the amino acid sequence of the precursor protein, it is unnecessary to provide the mechanics of processing as this information is natural and intrinsic to the expression system being utilized.

The Mature Form of VEGF-2 As Expressed and Secreted is an Inherent Feature of the Sequence of the Precursor Polypeptide

13. I have also been asked to comment on the characterization of the inherent features of the mature form of VEGF-2 as provided by Dr. Alitalo in portions of the file history of U.S. Patent No. 6,221,839, issued April 24, 2001, from U.S. Application No. 08/510,133, filed August 1, 1995, ("the Alitalo application"). I have reviewed: (a) Dr. Alitalo's Conditional Petition to

Reverse or Withdraw Adverse Priority Determination Pursuant to 37 C.F.R. § 1.181, dated June 11, 1997 (the "Petition" attached hereto as Exhibit C) and (b) a Declaration by Dr. Carl-Henrik Heldin, executed June 4, 1997 (the "Heldin Declaration" attached hereto as Exhibit D), both provided in the prosecution of the Alitalo application.

14. According to Dr. Alitalo, the *molecular weight* of the processed mature form of VEGF-2 as assessed by SDS-PAGE under reducing conditions is an inherent property of the molecule (*See* Petition page 7, line 31 to page 8, lines 24-26). Dr. Heldin is also in agreement with this principal, stating, "[l]ike all organic chemical compounds, polypeptides may be characterized by any of several inherent physical properties, such as *molecular formula* and *molecular weight*. Such physical properties are inherent characteristics of organic molecules in that they are intrinsic properties of the molecules" (*See* Heldin Declaration § 10, at page 6, lines 12-13, emphasis added).

15. Dr. Alitalo also characterizes the processing of the precursor form of the VEGF-2 polypeptide to a *partial amino acid sequence of its precursor* as an inherent property of that polypeptide (*See* Petition page 8, lines 24-26). Dr. Heldin provides a similar characterization, in that the secreted mature form of VEGF-2 has a *partial amino acid sequence of its precursor form* and an apparent 23kD molecular weight, both of which are inherent features, intrinsic to the secreted form of the polypeptide (*See* Heldin Declaration § 11, at page 6, lines 28-30).

16. Dr. Alitalo characterizes the ability of the secreted form to contain those residues required for *activity* as another inherent feature of the VEGF-2 polypeptide (*See* Petition page 15, lines 7-15). Dr. Heldin further expands on Dr. Alitalo's characterization, stating that the secreted mature form of VEGF-2 comprises a portion of the amino acid sequence of the precursor form of VEGF-2 which binds to the Flt4 receptor tyrosine kinase and stimulates

phosphorylation thereof, and that these *activities* are inherent properties of the polypeptide (*See* Heldin Declaration § 11, at page 8, lines 2-6).

17. Thus, as Drs. Alitalo and Heldin have concluded, I would also conclude that the capacity of the precursor form of VEGF-2 to be processed to a mature form that retains a portion of the precursor amino acid sequence is a natural and intrinsic property of that amino acid sequence. It is the precursor form of the polypeptide that intrinsically contains the information required for the cell's machinery to process the protein to its mature form. Indeed, the capacity of the precursor form of VEGF-2 to be naturally processed to its mature form is an inherent property of that polypeptide, as confirmed by the experiments described in Dr. Powers' Declaration as discussed above in paragraphs 6-9. The molecular weight of the resulting processed form is an inherent property of that polypeptide as it is processed by the cell from which it is expressed. The resulting processed mature form possesses biological activity which is also an inherent property of that polypeptide.

Conclusion

18. In my opinion, the experimental evidence provided in Dr. Power's Declaration confirms the teachings of the HGS patent specification, demonstrating that the expression of the construct encoding the 350 amino acid sequence as set forth in the patent specification results in a secreted mature form of VEGF-2. Furthermore, these results confirm that the construct encoding the 350 amino acid sequence contains sufficient information to be naturally and correctly processed by the cell resulting in a mature processed VEGF-2 protein.

19. Following the teachings of the HGS patent specification, as demonstrated by Dr. Power's Declaration, I or a molecular biologist would recognize that the 350 amino acid sequence of VEGF-2 has the capacity to be processed to a mature form of VEGF-2 by the cell. The capacity of the precursor form of

VEGF-2 to be processed to a mature form is an intrinsic property of that polypeptide.

20. Therefore, a molecular biologist provided with the teaching of the HGS patent specification would be able to express the precursor form of VEGF-2 as its naturally processed mature form. Further, a molecular biologist provided with the teaching of the HGS patent specification would recognize that the biological activity and molecular weight of the resulting processed mature form of VEGF-2 are intrinsic and natural properties of that molecule. Thus, based on the teachings of the HGS patent specification, a molecular biologist would be able to identify and obtain a mature processed form of VEGF-2.

AND I declare further that all statements made in this Declaration of my own are true in every particular, and that all statements made on information and belief are believed to be true.

Date: November 29, 2001

Stuart Aaronson

6/28/00

CURRICULUM VITAE

Name: Stuart A. Aaronson

Date and Place of Birth: February 28, 1942, Mt. Clemens, Michigan

Citizenship: U.S.A.

Marital Status: Married, three children

Education and Training:

1959-1962	B.S. (Chemistry; summa cum laude), University of California, Berkeley
1962-1966	M.D., University of California Medical School, San Francisco
1965-1966	Fellowship, Dept. of Biochemistry, University of Cambridge, Cambridge, United Kingdom
1966-1967	Intern, Medicine, Moffitt Hospital, San Francisco

Brief Chronology of Employment:

1967-1969	Staff Associate, Viral Carcinogenesis Branch, National Cancer Institute, Bethesda, MD
1969-1970	Senior Staff Fellow, Viral Carcinogenesis Branch
1970-1977	Head, Molecular Biology Section, Viral Carcinogenesis Branch
1977-1993	Chief, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland
1993-	Director, Ruttenberg Cancer Center, Mount Sinai Medical Center, New York, NY & Jane B. and Jack R. Aron, Professor of Neoplastic Diseases

Medical Licenses

New York
Virginia

Honors and Awards:

1962	Phi Beta Kappa
1966	Alpha Omega Alpha
1982	Rhoads Memorial Award
1982	PHS Meritorious Service Medal
1989	Paul Ehrlich Award
1989	PHS Distinguished Service Medal
1990	Milken Award
1991	Chirone Prize
1991	Harvey Lecture

1991 Wadsworth Memorial Foundation Award

Societies:

American Society for Microbiology
American Association for the Advancement of Science
Society for Experimental Biology and Medicine
American Association for Cancer Research, Inc.
American Society for Virology, Inc.

Memberships and Affiliations:

1975-1978 Member, Viral Cancer Program Coordinating Committee
1975-1976 Ad Hoc Member, Experimental Virology Study Section, NIH
1975-1978 Member, Viral Oncology Scientific Advisory Committee for FCRC
1976-1980 Member, Experimental Virology Study Section, NIH
1977- Member, Editorial Board, International Journal of Cancer
1977-1986 Associate Editor, Journal of the National Cancer Institute
1980-1985 Editorial Advisory Board, Biochimica et Biophysica Acta (BBA Reviews on Cancer)
1981- Associate Editor, Cancer Research
1983- Executive Committee, Duke Comprehensive Center, Duke University Medical Center
1984 Mott Selection Committee, General Motors Cancer Research Foundation
1984- Advisory Committee, Maimonides Conferences on Cancer Research
1984-1990 Editorial Board, Virus Research
1984-1987 Scientific Advisory Committee, American Cancer Society
1985-1987 External Scientific Review Committee, Comprehensive Center, The University of Alabama in Birmingham
1985- Editorial Advisory Board, Cancer and Metastasis Reviews
1985- Editorial Board, Cancer Reviews
1985-1989 Councillor, Society for Experimental Biology and Medicine
1985-1990 Extramural Advisory Board, Cancer Center, The University of Arizona
1986 Program Chairman, American Association of Cancer Research
1986 Co-organizer, Princess Takamatsu Symposium
1986- Guest Editor, Japanese Journal of Cancer Research (Gann)
1986- Editorial Board, Environmental and Occupational Health Sciences
1986-1987 Member, Advisory Committee, American Type Culture Collection
1987-1989 Editorial Advisory Board, Molecular Endocrinology
1987- Editorial Board, Oncogene
1988-1989 Advisory Editorial Board, ISI Atlas of Science: Biochemistry
1988- Member, Blood Services Scientific Council, American Red Cross
1989-1991 Editorial Board, Cancer Communications

1989-1992 Editorial Board, The New Biologist
1989 Visiting Professor, University of Texas, San Antonio
1990- Advisory Board, BBA Reviews on Cancer, Biochimica et Biophysica Acta
1990- General Motors Visiting Professor, University of Wisconsin-Madison Medical School
1990- Visiting Professor, Jonsson Comprehensive Cancer Center, University of California, Los Angeles
1992- Editorial Board, Intl. Journal of Oncology
1992- Editorial Board, Oncology Research
1992 Scientific Advisory Board, - Georgetown Univ – Breast Ca SPORE
1993-1995 Editorial Advisory Board, Molecular Aspects of Medicine
1994- International Advisory Board, Tumori
1995-1996 Vice President, Harvey Society
1995- External Scientific Advisory Committee, UCLA Oral Cancer Center
1996-1997 President, Harvey Society
1997-1998 Counselor, Harvey Society
1998 Member, Public Relations and Communications Committee, AACR
1998 Member, The National Neurofibromatosis Foundation Research Advisory Board

Research Interests:

Molecular genetics of cancer; retrovirology; cellular growth regulation by growth factors and their receptors.

Patents:

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COMMONWEALTH OF AUSTRALIA

IN THE MATTER OF: Australian Patent Application 696764 (73941/94). In the name of: Human Genome Sciences Inc.

-and-

IN THE MATTER OF: Opposition thereto by Ludwig Institute for Cancer Research, under Section 59 of the Patents Act.

STATUTORY DECLARATION

I, Susan Power of Cell & Molecular Technologies, Inc., Phillipsburg, New Jersey, United States of America, declare as follows:

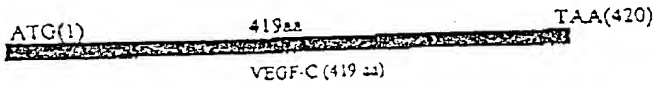
1. I am currently a Senior Molecular Biologist for Cell & Molecular Technologies, Phillipsburg, New Jersey. I have held this position since July, 1999. Prior to that appointment I was a post-doctorate research scientist, studying the molecular biology of retenoids and their role in limb bud development, at the University of Wisconsin, Madison, Wisconsin for two years. Prior to that appointment, I was a post-doctorate research scientist, studying the molecular biology of the transcription factor vHNF1 at the Pasteur Institute in Paris, France for four years. I received my Ph.D. in 1991 in Microbiology, from the National University of Ireland, Galway, Ireland.

2. The Patent Attorneys for Human Genome Sciences (HGS) requested that I perform the following experiments in order to determine whether the 350 amino acid form of VEGF-2 (corresponding to residues 70 to 419 of the 419 amino acid form of VEGF-2) fused in frame with a heterologous signal sequence would result in the expression and secretion of the protein from eukaryotic cells. They have also requested that all experiments that I conducted employ techniques routinely available by March, 1994. I have done this and the experiments I have conducted are described herein. Unless I state otherwise, all methods used herein were available prior to March, 1994.

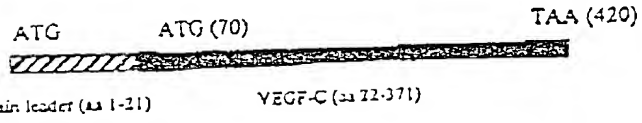
3. To determine whether the 350 amino acid form of VEGF-2 could be secreted from cells when attached to a heterologous signal sequence, I transfected eukaryotic cells with expression vectors encoding the 419 amino acid form of VEGF-2, or the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence. I grew the transfected cells under conditions to allow the cells to express the gene products encoded by the vectors. At various time points, I collected both cell lysates and culture medium and assayed for the presence of VEGF-2, in order to determine if the cells were secreting VEGF-2. The presence of VEGF-2 in either the cell lysates or culture medium was determined by Western Blot analysis of the samples using a polyclonal antibody to VEGF-2, which recognizes both the precursor form and the processed form of VEGF-2

4. The design of the expression vectors used in the study is as follows:

419 amino acid form of VEGF-2 (followed by a STOP codon at position 420):



350amino acid form of VEGF-2 linked to a heterologous signal sequence (followed by a STOP codon at position 420):



5. The nucleotide sequences encoding the 419 and the 350 amino acid forms of VEGF-2 were obtained directly from the American Type Culture Collection (ATCC). ATCC Deposit No. 97149 contains the nucleotide sequence encoding the 419 amino acid form of VEGF-2. ATCC Deposit No. 75698 contains the nucleotide sequence encoding the 350 amino acid form of VEGF-2. The nucleotide sequence encoding the 419 amino acid form of VEGF-2

was engineered to be flanked by an Eco RI site at the 5'end and a Not I site at its 3'end. The second construct contained the nucleotide sequence encoding the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, Ig k-chain leader signal sequence, a recognized signal sequence which was available as of March, 1994, and was engineered to contain a Bam HI site at its 5'end and a Not I site at its 3' end. The sequence of each of these constructs was confirmed to be correct and is detailed in Appendix I, attached hereto.

6. Each VEGF-2 construct was subcloned into the expression vector pCMV-I (see attached plasmid map, attached hereto as Appendix II), so that the VEGF-2 sequences were under the control of a CMV-I promoter, a promoter routinely used as of March, 1994. The 419 amino acid form of VEGF-2 was subcloned into the Eco RI/Not I sites of pCMV-I, while the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, was subcloned into the Bam HI/Not I sites of pCMV-I (see plasmid map, Appendix II).

7. The two VEGF-2 constructs were transiently transfected in duplicate, using the lipofectin method, comparable methods were routinely used as of March, 1994, into the Human Embryonic Kidney cell line, HEK-293 tsA-0, a cell line which was also routinely used as of March, 1994. As a control for transfection efficiency, each construct was co-transfected with the vector pCMV-β-gal. The efficiency of transfection was determined by β-gal staining 48 hours after transfection. As a negative control the vector pCMV-I without an insert was transfected in parallel.

8. The transfection design is as follows:
10 dishes transfected with: pCMV-I-VEGF-419;
10 dishes transfected with: pCMV-I-signal sequence-VEGF-350;
10 dishes transfected: pCMV-I ;
2 dishes transfected with: pCMV-I-VEGF-419 + pCMV-β-gal; and
2 dishes transfected with: pCMV-I-signal sequence-VEGF-350 + pCMV-β-gal.

9. After transfection, DMEM medium containing 3% serum was added to the cells. Aliquots of cell extracts and conditioned medium were prepared from each transfection at: T_0 hours, T_{16} hours, T_{24} hours, T_{48} hours and T_{72} hours, in duplicate.

10. At the time of harvesting the cells and medium were treated as follows:
Medium: Harvested medium was concentrated 3 fold using Centricon 10 concentrator devices. One volume of 2 x PAGE loading dye was added to each sample.
Cell Extracts: The cells were harvested by trypsinization and collected by centrifugation. The cell pellet was resuspended and lysed in 250µl of 1x PAGE loading buffer.

11. To determine the transfection efficiency, dishes transfected with the pCMV-β-gal construct were fixed and stained for β-gal activity 48 hours after transfection. All dishes were found to have the same percentage of transfected cells (70%).

12. Each protein sample was subjected to Western analysis as outlined below. Prior to loading on to a 12% (w/v) Tris-Glycine SDS-Polyacrylamide gel, the samples were boiled for 5 minutes and cooled on ice. The two end lanes of each gel contained the appropriately sized molecular weight markers to estimate the migration rate of proteins predicted to run in the 16 to 85 kDa size range. The samples were electrophoresed according to standard conditions.

13. Following electrophoresis, the samples were transferred to a PVDF membrane. Each membrane was blocked by a one hour incubation in phosphate buffered saline (PBS) containing 3% Bovine Serum Albumin (BSA). The blot was then incubated at 4°C in PBS containing 0.1% BSA and 500ng/ul of purified rabbit anti-VEGF-2 antibody, a polyclonal antibody which recognizes the precursor form and the processed form of VEGF-2. After three 5 minute washes in PBS containing 0.1% Tween, the blot was incubated for 1 hour in PBS containing 0.1% BSA and a 1:3000 dilution of Goat Anti-Rabbit IgG

Horse Radish Peroxidase (HRP) conjugated antibody. The blot was washed three times for 5 minutes in PBS containing 0.1% BSA. The blot was developed with 2ml/blot of ECL detection reagent (obtained from Amersham) for one minute and then exposed directly to Polaroid films for approximately 5 seconds.

14. The result of the experiment is shown in Figure 1, attached hereto. The samples included in the figure are as follows:

Immunoblot analysis of VEGF derivatives transiently expressed in HEK293T cells

Lane	Pellet/Supernn.	350aa -signal / 419aa	T (h) post-transfection
Gel 1			
1	P	419	24
2	S	419	24
3	P	350-signal	24
4	S	350-signal	24
5	P	350-signal	24
6	S	350-signal	24
7	P	negative control	24
8	S	positive control	48
Gel 2			
9	P	419	48
10	S	419	48
11	P	419	48
12	S	419	48
13	P	350-signal	48
14	S	350-signal	48

15	P	350-signal	48
16	S	350-signal	48
17	P	negative control	
18	S	negative control	
Gel 3			
19	P	419	72
20	S	419	72
21	P	419	72
22	S	419	72
23	P	350-signal	72
24	S	350-signal	72
25	S	350-signal	72
26	P	350-signal	72

15. The Western Blot analysis indicates that a doublet of approximately 30kDa was present in the medium collected from the transfection of both the 419 amino acid form of VEGF-2 and the 350 amino acid-VEGF-2 signal sequence constructs (see Figure 1). The secreted protein was visible beginning at 16 hours after transfection. The secreted product from cells containing the 419 amino acid construct and the 350 amino acid-VEGF2 signal sequence construct are approximately the same size.

AND I declare that all the statements made in this Declaration are of my own are true in every particular, and that all statements made on information and belief are believed to be true.

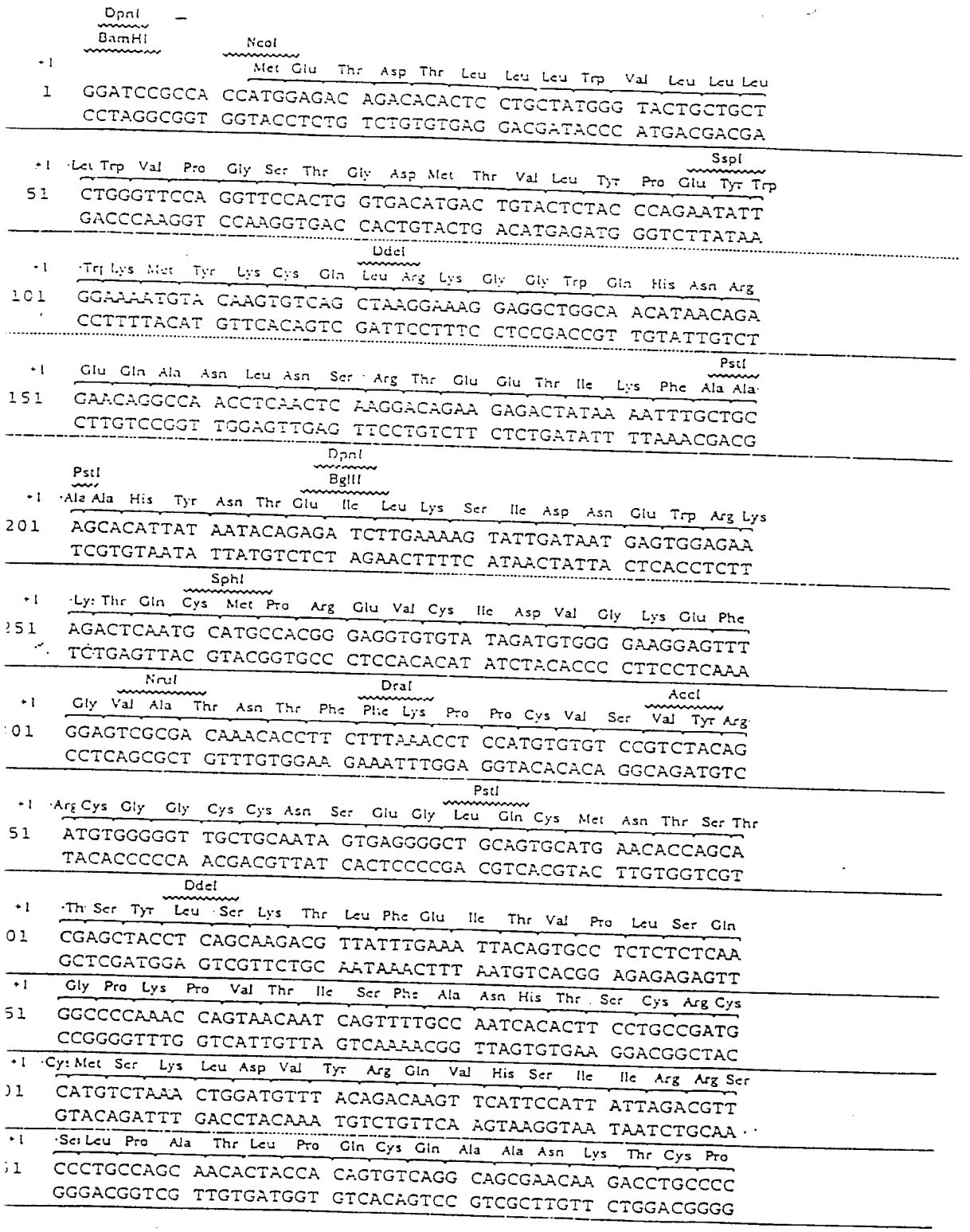
Sworn by the said Susan Power, Susan Power at Phillipsburg, New Jersey, on this 5th day of December 2000;

before me Maryann White.

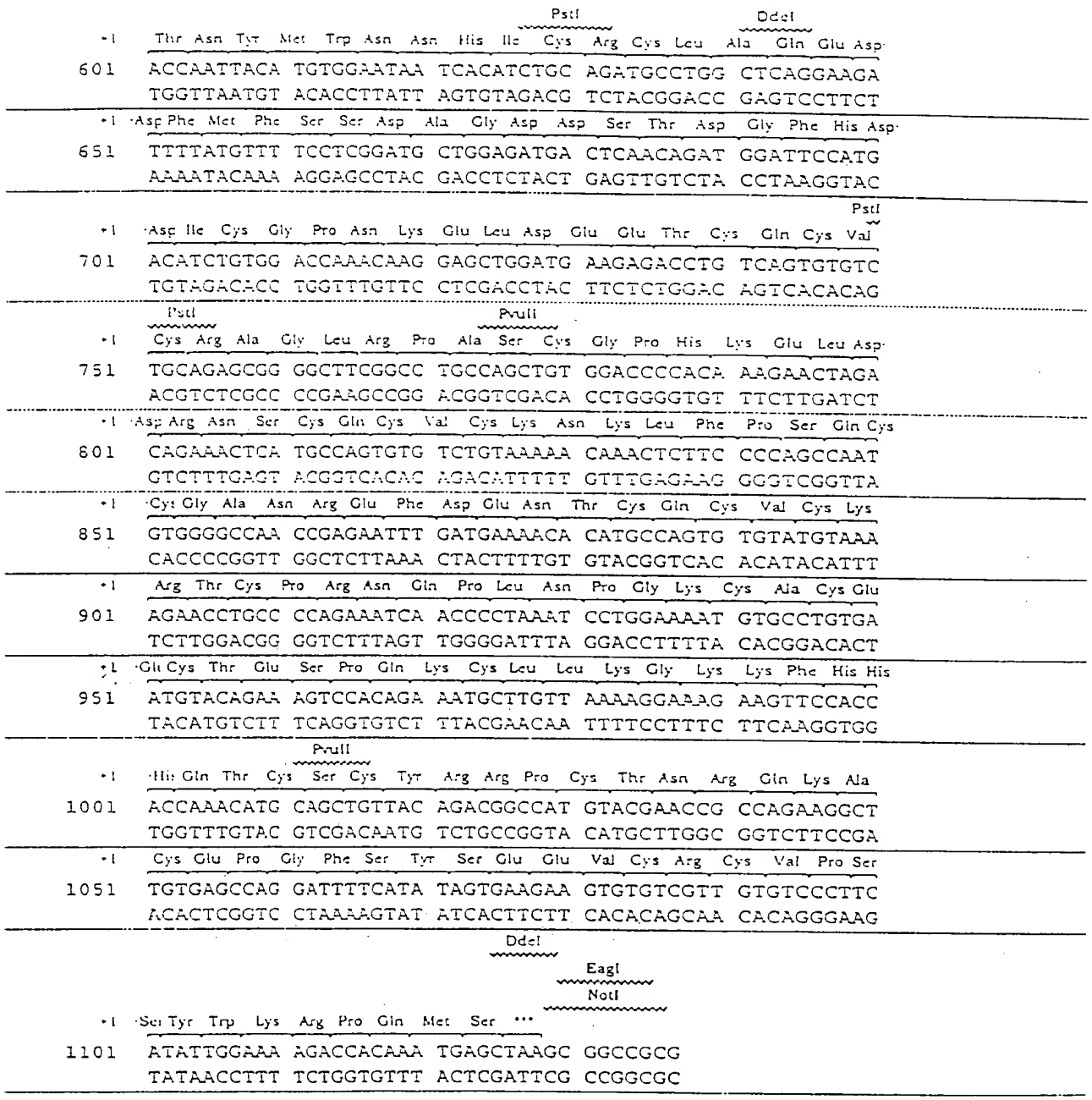
Notary Public

MARYANN WHITE
NOTARY PUBLIC, State of New York
No. 4883761
Qualified in Nassau County
Certification Filed in New York County
Commission Expires January 26, 2001

VEGF-2 350aa +Signal Sequence:



Appendix I


PstI
DdeI
+1 Thr Asn Tyr Met Trp Asn Asn His Ile Cys Arg Cys Leu Ala Gln Glu Asp
601 ACCAATTACA TGTGGAATAA TCACATCTGC AGATGCCTGG CTCAGGAAGA
TGGTTAATGT ACACCTTATT AGTGTAGACG TCTACGGACC GAGTCCTTCT
+1 Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp Gly Phe His Asp
651 TTTTATGTTT TCCTCGGATG CTGGAGATGA CTCAACAGAT GGATTCCATG
AAAATACAAA AGGAGCCTAC GACCTCTACT GAGTTGTCTA CCTAAGGTAC
PstI
+1 Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys Val
701 ACATCTGTGG ACCAAACAAG GAGCTGGATG AAGAGACCTG TCAGTGTGTC
TGTAGACACC TGGTTTGTTC CTCGACCTAC TTCTCTGGAC AGTCACACAG
PstI
PvuII
+1 Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro His Lys Glu Leu Asp
751 TGCAGAGCGG GGCTTCGGCC TGCCAGCTGT GGACCCCACA AAGAACTAGA
ACGTCTCGCC CCGAAGCCGG ACGGTCGACA CCTGGGGTGT TTCTTGATCT
+1 Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Ser Gln Cys
801 CAGAAACTCA TGCCAGTGTG TCTGTAAAAA CAAACTCTTC CCCAGCCAAT
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+1 Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala Cys Glu
901 AGAACCTGCC CCAGAAATCA ACCCCTAAAT CCTGGAAAAT GTGCCTGTGA
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+1 Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly Lys Lys Phe His His
951 ATGTACAGAA AGTCCACAGA AATGCTTGTT AAAAGGAAAG AAGTTCCACC
TACATGTCTT TCAGGTGTCT TTACGAACAA TTTTCCTTTC TTCAAGGTGG
PvuII
+1 His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr Asn Arg Gln Lys Ala
1001 ACCAAACATG CAGCTGTTAC AGACGGCCAT GTACGAACCG CCAGAAGGCT
TGGTTTGTAC GTCGACAATG TCTGCCGGTA CATGCTTGGC GGTCTTCCGA
+1 Cys Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser
1051 TGTGAGCCAG GATTTTCATA TAGTGAAGAA GTGTGTCGTT GTGTCCCTTC
ACACTCGGTC CTAAAAGTAT ATCACTTCTT CACACAGCAA CACAGGGAAG
DdeI
EagI
NotI
+1 Ser Tyr Trp Lys Arg Pro Gln Met Ser ...
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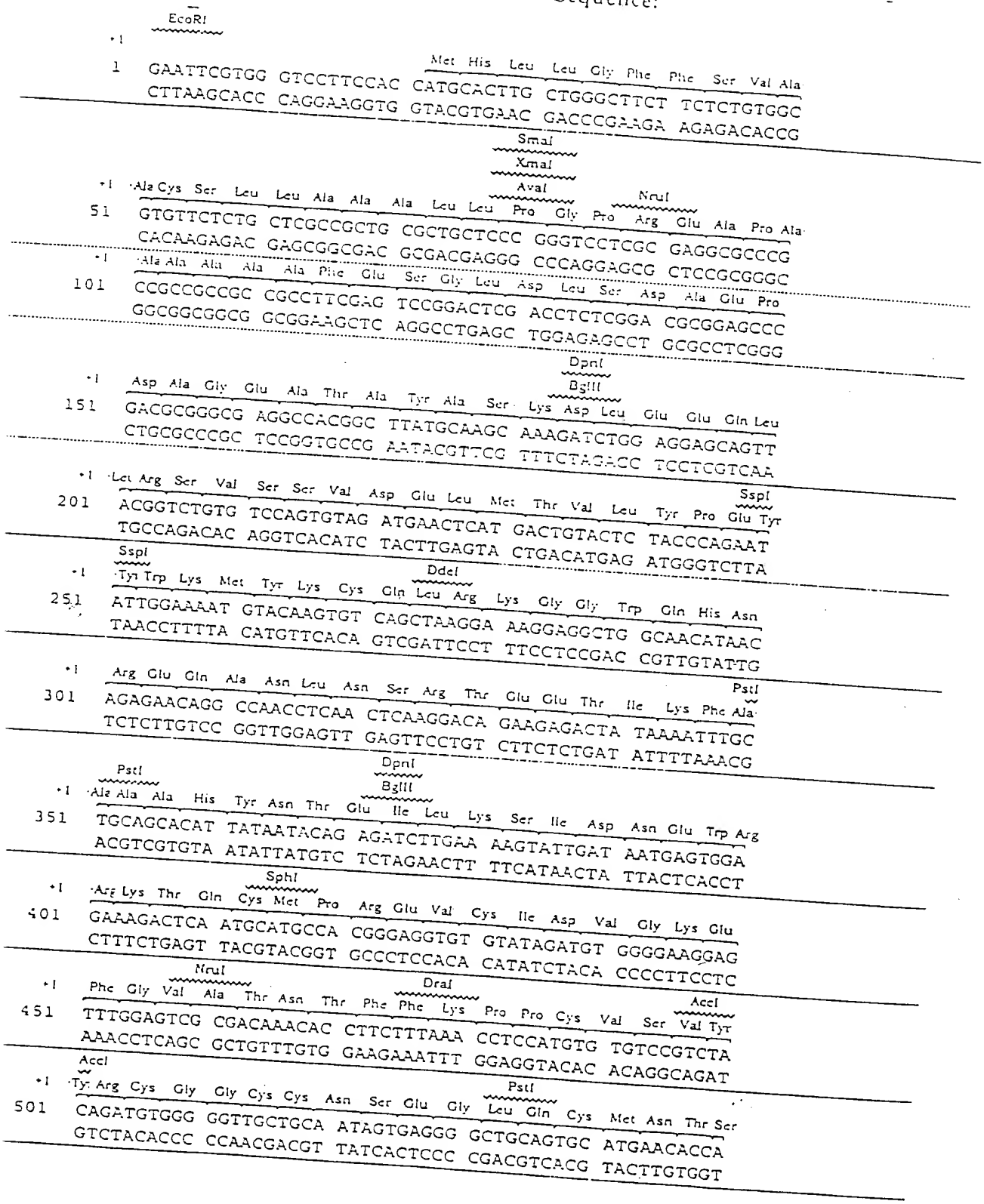
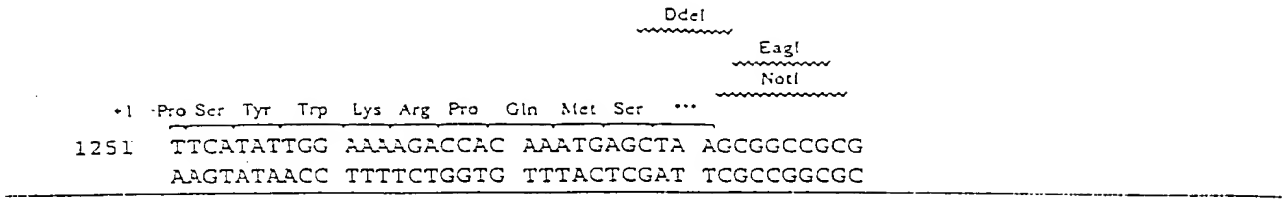
TATAACCTTT TCTGGTGTTT ACTCGATTCG CCGGCGC

VEGF-2 419aa Sequence:




DdeI
EagI
NotI
+1 Pro Ser Tyr Trp Lys Arg Pro Gln Met Ser ...
1251 TTCATATTGG AAAAGACCAC AAATGAGCTA AGCGGCCGCG
AAGTATAACC TTTTCTGGTG TTTACTCGAT TCGCCGGCGC

STRAIN SHEET

STRAIN CMV I STORAGE NUMBER 11:64

PEDIGREE:

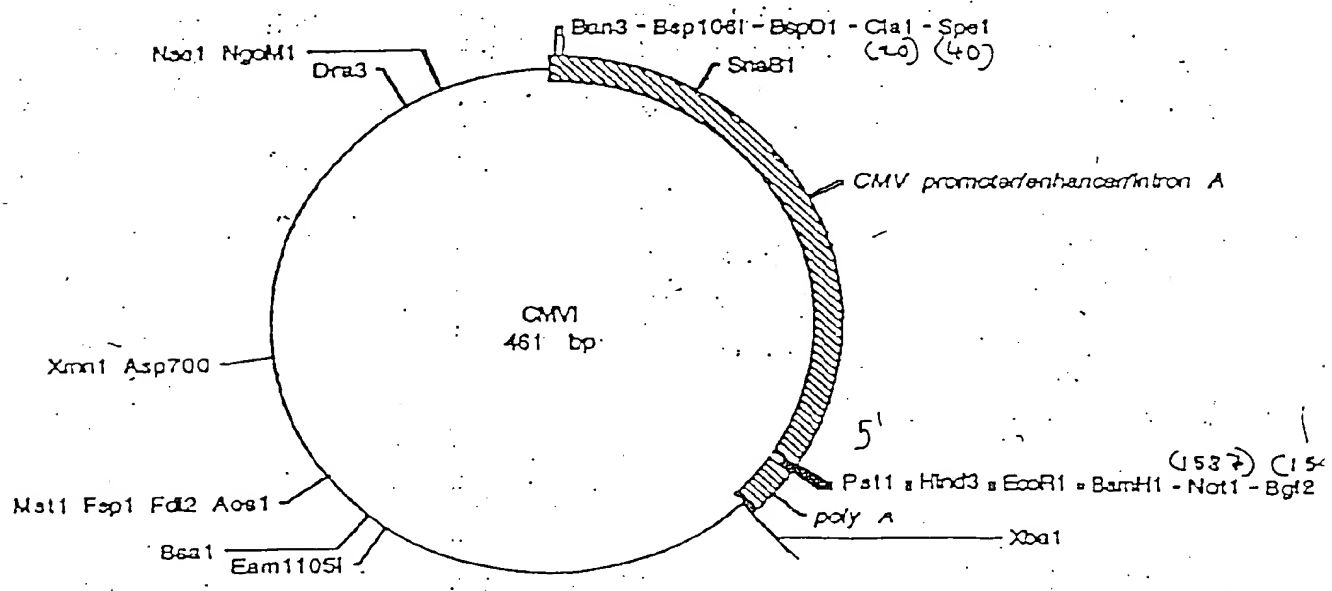
-CMVI was constructed in the pSV7 (nee pSG5 from Stratagene, with an expanded polylinker) backbone by replacing the SV40 promoter from pSV7 with the CMV promoter/enhancer/intron, via 5' Sal I/Xho I (sites destroyed) and 3' Hind III.

-CMVI is 4613 bp.

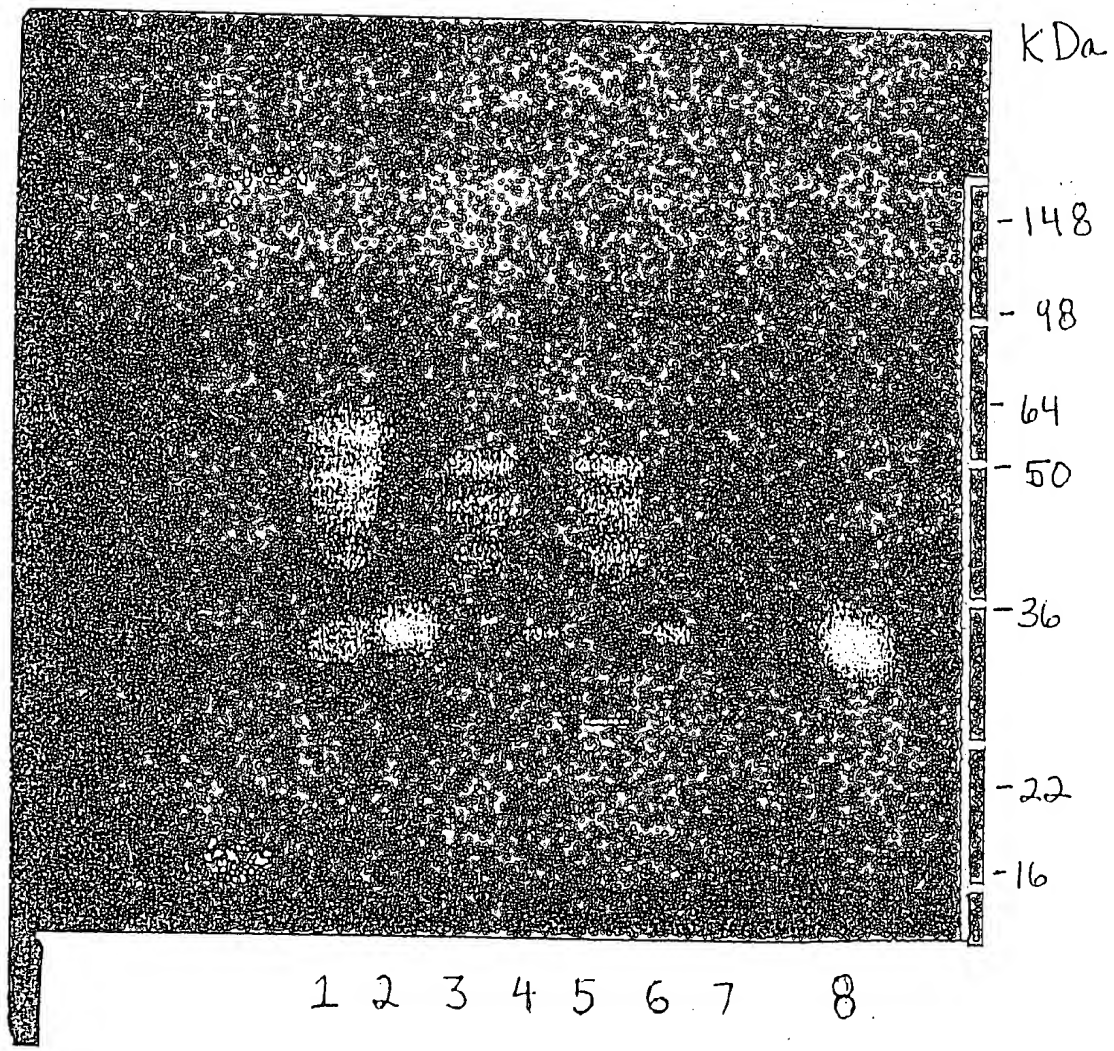
-CMVI uses ampicillin resistance.

-CMV promoter/enhancer + Intron A: nt #'s 1-1566.
-Polylinker: nt #'s 1566-1597 (5'- Hind III - Eco RI - Bam HI - Not I - Bgl II - 3').
-SV40 polyA addition sequence: nt #'s 1598-1745.
-If for some reason you want to remove the SV40 polyA addition sequence, you can cut with either Sal I or Xba I (these 2 sites border the 3' end of this sequence).

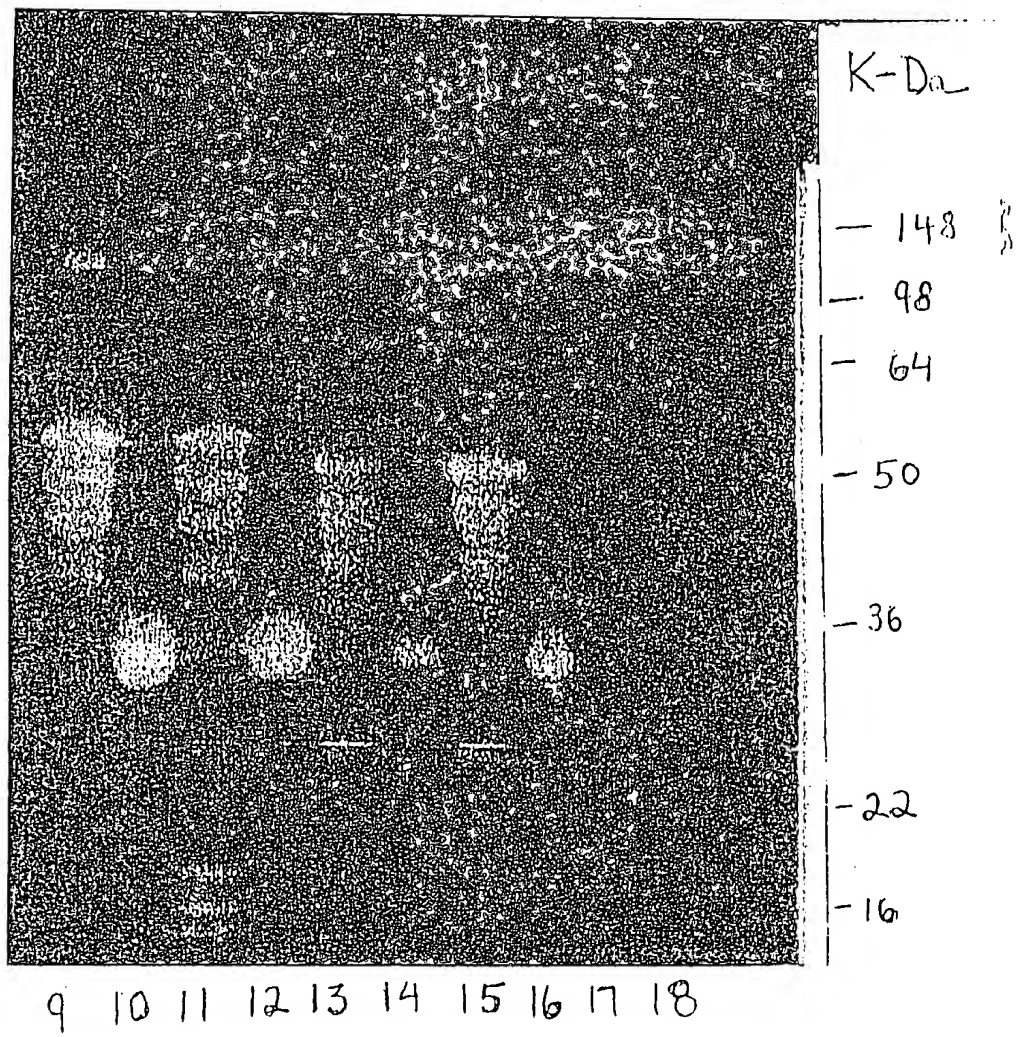
GROWTH REQUIREMENTS:



Appendix II



Power Declaration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50
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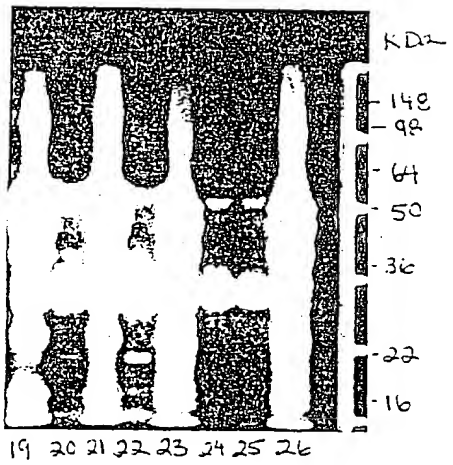
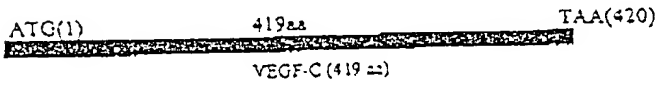


Power Declaration
Figure 1 - Gel 3

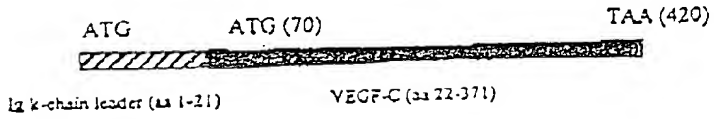
3. To determine whether the 350 amino acid form of VEGF-2 could be secreted from cells when attached to a heterologous signal sequence, I transfected eukaryotic cells with expression vectors encoding the 419 amino acid form of VEGF-2, or the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence. I grew the transfected cells under conditions to allow the cells to express the gene products encoded by the vectors. At various time points, I collected both cell lysates and culture medium and assayed for the presence of VEGF-2, in order to determine if the cells were secreting VEGF-2. The presence of VEGF-2 in either the cell lysates or culture medium was determined by Western Blot analysis of the samples using a polyclonal antibody to VEGF-2, which recognizes both the precursor form and the processed form of VEGF-2

4. The design of the expression vectors used in the study is as follows:

419 amino acid form of VEGF-2 (followed by a STOP codon at position 420):



350amino acid form of VEGF-2 linked to a heterologous signal sequence (followed by a STOP codon at position 420):



5. The nucleotide sequences encoding the 419 and the 350 amino acid forms of VEGF-2 were obtained directly from the American Type Culture Collection (ATCC). ATCC Deposit No. 97149 contains the nucleotide sequence encoding the 419 amino acid form of VEGF-2. ATCC Deposit No. 75698 contains the nucleotide sequence encoding the 350 amino acid form of VEGF-2. The nucleotide sequence encoding the 419 amino acid form of VEGF-2

was engineered to be flanked by an Eco RI site at the 5'end and a Not I site at its 3'end. The second construct contained the nucleotide sequence encoding the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, Ig k-chain leader signal sequence, a recognized signal sequence which was available as of March, 1994, and was engineered to contain a Bam HI site at its 5'end and a Not I site at its 3' end. The sequence of each of these constructs was confirmed to be correct and is detailed in Appendix I, attached hereto.

6. Each VEGF-2 construct was subcloned into the expression vector pCMV-I (see attached plasmid map, attached hereto as Appendix II), so that the VEGF-2 sequences were under the control of a CMV-I promoter, a promoter routinely used as of March, 1994. The 419 amino acid form of VEGF-2 was subcloned into the Eco RI/Not I sites of pCMV-I, while the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, was subcloned into the Bam HI/Not I sites of pCMV-I (see plasmid map, Appendix II).

7. The two VEGF-2 constructs were transiently transfected in duplicate, using the lipofectin method, comparable methods were routinely used as of March, 1994, into the Human Embryonic Kidney cell line, HEK-293 tsA-0, a cell line which was also routinely used as of March, 1994. As a control for transfection efficiency, each construct was co-transfected with the vector pCMV-β-gal. The efficiency of transfection was determined by β-gal staining 48 hours after transfection. As a negative control the vector pCMV-I without an insert was transfected in parallel.

8. The transfection design is as follows:
10 dishes transfected with: pCMV-I-VEGF-419;
10 dishes transfected with: pCMV-I-signal sequence-VEGF-350;
10 dishes transfected: pCMV-I ;
2 dishes transfected with: pCMV-I-VEGF-419 + pCMV-β-gal; and
2 dishes transfected with: pCMV-I-signal sequence-VEGF-350 + pCMV-β-gal.

9. After transfection, DMEM medium containing 3% serum was added to the cells. Aliquots of cell extracts and conditioned medium were prepared from each transfection at: T_0 hours, T_{16} hours, T_{24} hours, T_{48} hours and T_{72} hours, in duplicate.

10. At the time of harvesting the cells and medium were treated as follows:
Medium: Harvested medium was concentrated 3 fold using Centricon 10 concentrator devices. One volume of 2 x PAGE loading dye was added to each sample.
Cell Extracts: The cells were harvested by trypsinization and collected by centrifugation. The cell pellet was resuspended and lysed in 250µl of 1x PAGE loading buffer.

11. To determine the transfection efficiency, dishes transfected with the pCMV-β-gal construct were fixed and stained for β-gal activity 48 hours after transfection. All dishes were found to have the same percentage of transfected cells (70%).

12. Each protein sample was subjected to Western analysis as outlined below. Prior to loading on to a 12% (w/v) Tris-Glycine SDS-Polyacrylamide gel, the samples were boiled for 5 minutes and cooled on ice. The two end lanes of each gel contained the appropriately sized molecular weight markers to estimate the migration rate of proteins predicted to run in the 16 to 85 kDa size range. The samples were electrophoresed according to standard conditions.

13. Following electrophoresis, the samples were transferred to a PVDF membrane. Each membrane was blocked by a one hour incubation in phosphate buffered saline (PBS) containing 3% Bovine Serum Albumin (BSA). The blot was then incubated at 4°C in PBS containing 0.1% BSA and 500ng/ul of purified rabbit anti-VEGF-2 antibody, a polyclonal antibody which recognizes the precursor form and the processed form of VEGF-2. After three 5 minute washes in PBS containing 0.1% Tween, the blot was incubated for 1 hour in PBS containing 0.1% BSA and a 1:3000 dilution of Goat Anti-Rabbit IgG

Horse Radish Peroxidase (HRP) conjugated antibody. The blot was washed three times for 5 minutes in PBS containing 0.1% BSA. The blot was developed with 2ml/blot of ECL detection reagent (obtained from Amersham) for one minute and then exposed directly to Polaroid films for approximately 5 seconds.

14. The result of the experiment is shown in Figure 1, attached hereto. The samples included in the figure are as follows:

Immunoblot analysis of VEGF derivatives transiently expressed in HEK293T cells

Lane	Pellet/Supernatant	350aa -signal / 419aa	T (h) post-transfection
Gel 1			
1	P	419	24
2	S	419	24
3	P	350-signal	24
4	S	350-signal	24
5	P	350-signal	24
6	S	350-signal	24
7	P	negative control	24
8	S	positive control	48
Gel 2			
9	P	419	48
10	S	419	48
11	P	419	48
12	S	419	48
13	P	350-signal	48
14	S	350-signal	48

15	P	350-signal	48
16	S	350-signal	48
17	P	negative control	
18	S	negative control	
Gel 3			
19	P	419	72
20	S	419	72
21	P	419	72
22	S	419	72
23	P	350-signal	72
24	S	350-signal	72
25	S	350-signal	72
26	P	350-signal	72

15. The Western Blot analysis indicates that a doublet of approximately 30kDa was present in the medium collected from the transfection of both the 419 amino acid form of VEGF-2 and the 350 amino acid-VEGF-2 signal sequence constructs (see Figure 1). The secreted protein was visible beginning at 16 hours after transfection. The secreted product from cells containing the 419 amino acid construct and the 350 amino acid-VEGF2 signal sequence construct are approximately the same size.

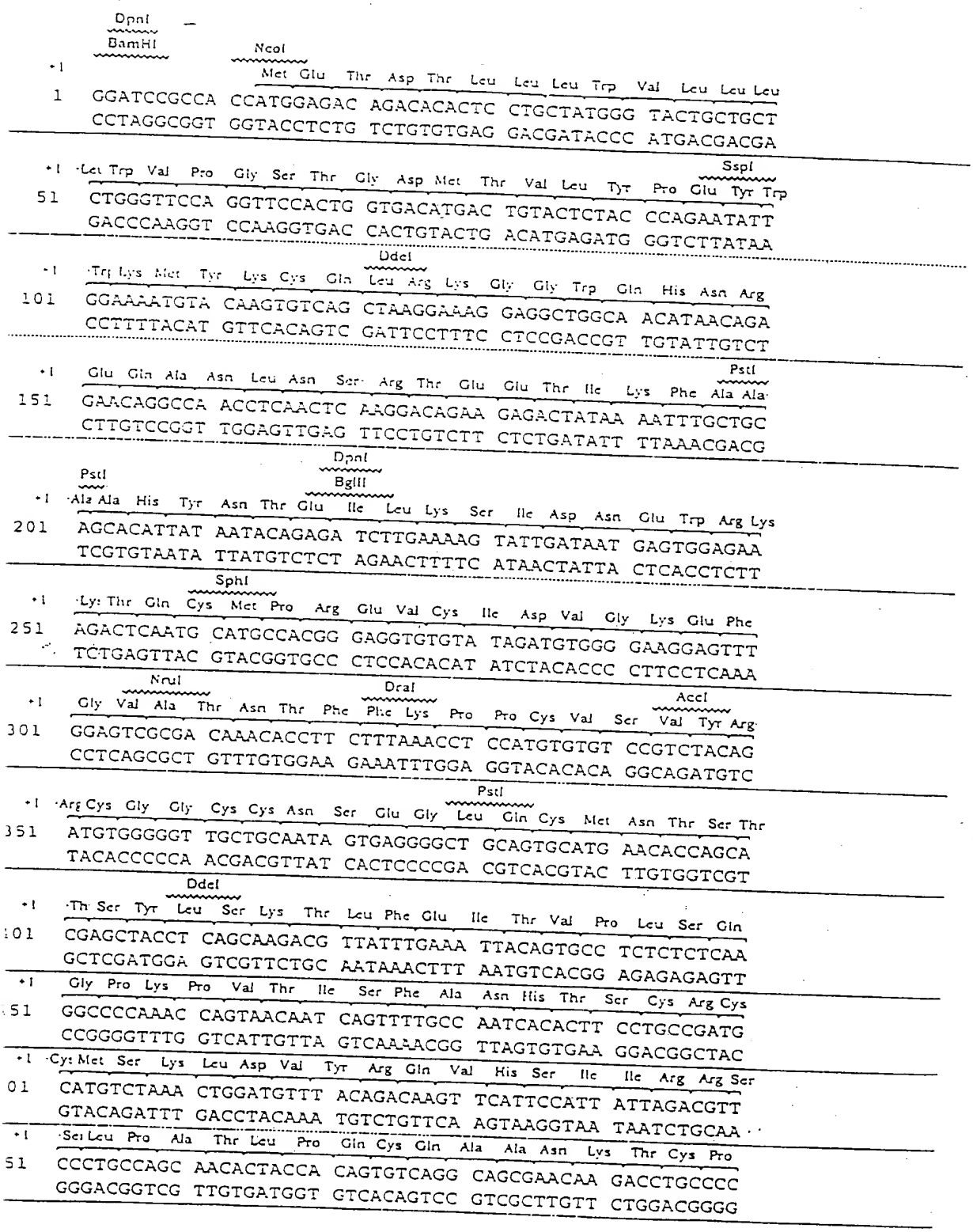
AND I declare that all the statements made in this Declaration are of my own are true in every particular, and that all statements made on information and belief are believed to be true.

Sworn by the said Susan Power, Susan Power at Phillipsburg, New Jersey, on this 13th day of December 2000; before me Maryann White.

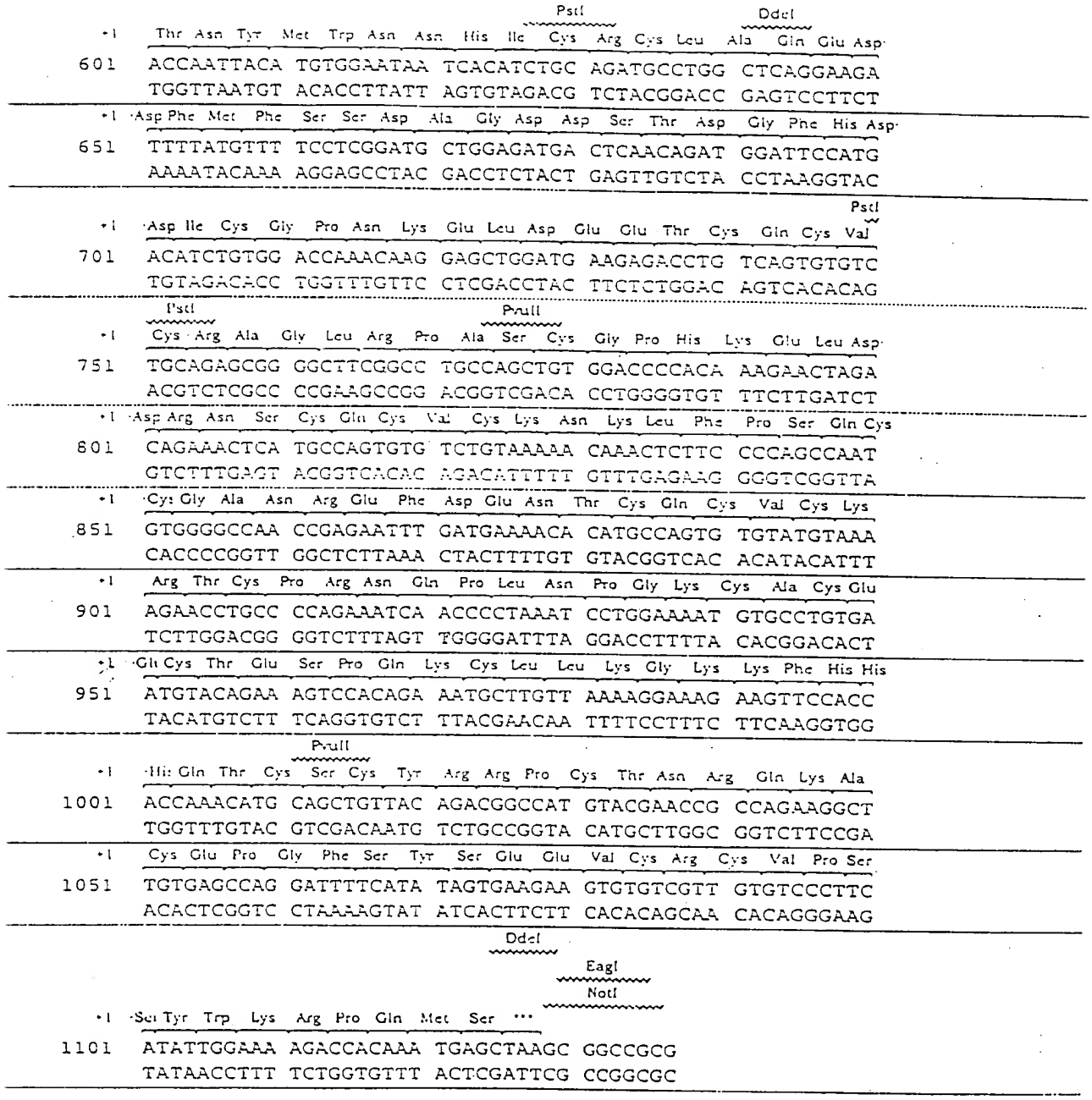
Notary Public

MARYANN WHITE
NOTARY PUBLIC, State of New York
No. 4883761
Qualified in Nassau County
Certification Filed in New York County
Commission Expires January 26, 2001

VEGF-2 350aa +Signal Sequence:



Appendix I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TGGTTAATGT ACACCTTATT AGTGTAGACG TCTACGGACC GAGTCCTTCT
+1 Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp Gly Phe His Asp
651 TTTTATGTTT TCCTCGGATG CTGGAGATGA CTCAACAGAT GGATTCCATG
AAAATACAAA AGGAGCCTAC GACCTCTACT GAGTTGTCTA CCTAAGGTAC
PstI
+1 Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys Val
701 ACATCTGTGG ACCAAACAAG GAGCTGGATG AAGAGACCTG TCAGTGTGTC
TGTAGACACC TGGTTTGTTC CTCGACCTAC TTCTCTGGAC AGTCACACAG
PstI
PvuII
+1 Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro His Lys Glu Leu Asp
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ACGTCTCGCC CCGAAGCCGG ACGGTCGACA CCTGGGGTGT TTCTTGATCT
+1 Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Ser Gln Cys
801 CAGAAACTCA TGCCAGTGTG TCTGTAAAAA CAAACTCTTC CCCAGCCAAT
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+1 Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys Val Cys Lys
851 GTGGGGCCAA CCGAGAATTT GATGAAAACA CATGCCAGTG TGTATGTAAA
CACCCCGGTT GGCTCTTAAA CTACTTTTGT GTACGGTCAC ACATACATTT
+1 Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala Cys Glu
901 AGAACCTGCC CCAGAAATCA ACCCCTAAAT CCTGGAAAAT GTGCCTGTGA
TCTTGGACGG GGTCTTTAGT TGGGGATTTA GGACCTTTTA CACGGACACT
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+1 Cys Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val Cys Arg Cys Val Pro Ser
1051 TGTGAGCCAG GATTTTCATA TAGTGAAGAA GTGTGTCGTT GTGTCCCTTC
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DdeI
EagI
NotI
+1 Ser Tyr Trp Lys Arg Pro Gln Met Ser ...
1101 ATATTGGAAA AGACCACAAA TGAGCTAAGC GGCCGCG
TATAACCTTT TCTGGTGTTT ACTCGATTCG CCGGCGC

VEGF-2 419aa Sequence:

```
                EcoRI
                                                                  Met  His  Leu  Leu  Gly  Phe  Phe  Ser  Val  Ala
   1  GAATTCGTGG GTCCTTCCAC CATGCACTTG CTGGGCTTCT TCTCTGTGGC
      CTTAAGCACC CAGGAAGGTG GTACGTGAAC GACCCGAAGA AGAGACACCG

                                                                  SmaI
                                                                  XmaI
                                                                  AvaI                NruI
  +1  Ala  Cys  Ser  Leu  Leu  Ala  Ala  Ala  Leu  Leu  Pro  Gly  Pro  Arg  Glu  Ala  Pro  Ala
  51  GTGTTCTCTG CTCGCCGCTG CGCTGCTCCC GGGTCCTCGC GAGGCGCCCG
      CACAAGAGAC GAGCGGCGAC GCGACGAGGG CCCAGGAGCG CTCCGCGGGC

  +1  Ala  Ala  Ala  Ala  Ala  Phe  Glu  Ser  Gly  Leu  Asp  Leu  Ser  Asp  Ala  Glu  Pro
 101  CCGCCGCCGC CGCCTTCGAG TCCGGACTCG ACCTCTCGGA CGCGGAGCCC
      GGCGGCGGCG GCGGAAGCTC AGGCCTGAGC TGGAGAGCCT GCGCCTCGGG

                                                                  DpnI
                                                                  BglII
  +1  Asp  Ala  Gly  Glu  Ala  Thr  Ala  Tyr  Ala  Ser  Lys  Asp  Leu  Glu  Glu  Gln  Leu
 151  GACGCGGGCG AGGCCACGGC TTATGCAAGC AAAGATCTGG AGGAGCAGTT
      CTGCGCCCGC TCCGGTGCCG AATACGTTCG TTTCTAGACC TCCTCGTCAA

                                                                                  SspI
  +1  Leu  Arg  Ser  Val  Ser  Ser  Val  Asp  Glu  Leu  Met  Thr  Val  Leu  Tyr  Pro  Glu  Tyr
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      TGCCAGACAC AGGTCACATC TACTTGAGTA CTGACATGAG ATGGGTCTTA

      SspI                                  DdeI
  +1  Tyr  Trp  Lys  Met  Tyr  Lys  Cys  Gln  Leu  Arg  Lys  Gly  Gly  Trp  Gln  His  Asn
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      TAACCTTTTA CATGTTCACA GTCGATTCCT TTCCTCCGAC CGTTGTATTG

                                                                                  PstI
  +1  Arg  Glu  Gln  Ala  Asn  Leu  Asn  Ser  Arg  Thr  Glu  Glu  Thr  Ile  Lys  Phe  Ala
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      TCTCTTGTCC GGTTGGAGTT GAGTTCCTGT CTTCTCTGAT ATTTTAAACG

                                                DpnI
      PstI                                      BglII
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                                    SphI
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                NruI                          DraI                                AccI
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      AAACCTCAGC GCTGTTTGTG GAAGAAATTT GGAGGTACAC ACAGGCAGAT

      AccI                                                  PstI
  +1  Tyr  Arg  Cys  Gly  Gly  Cys  Cys  Asn  Ser  Glu  Gly  Leu  Gln  Cys  Met  Asn  Thr  Ser
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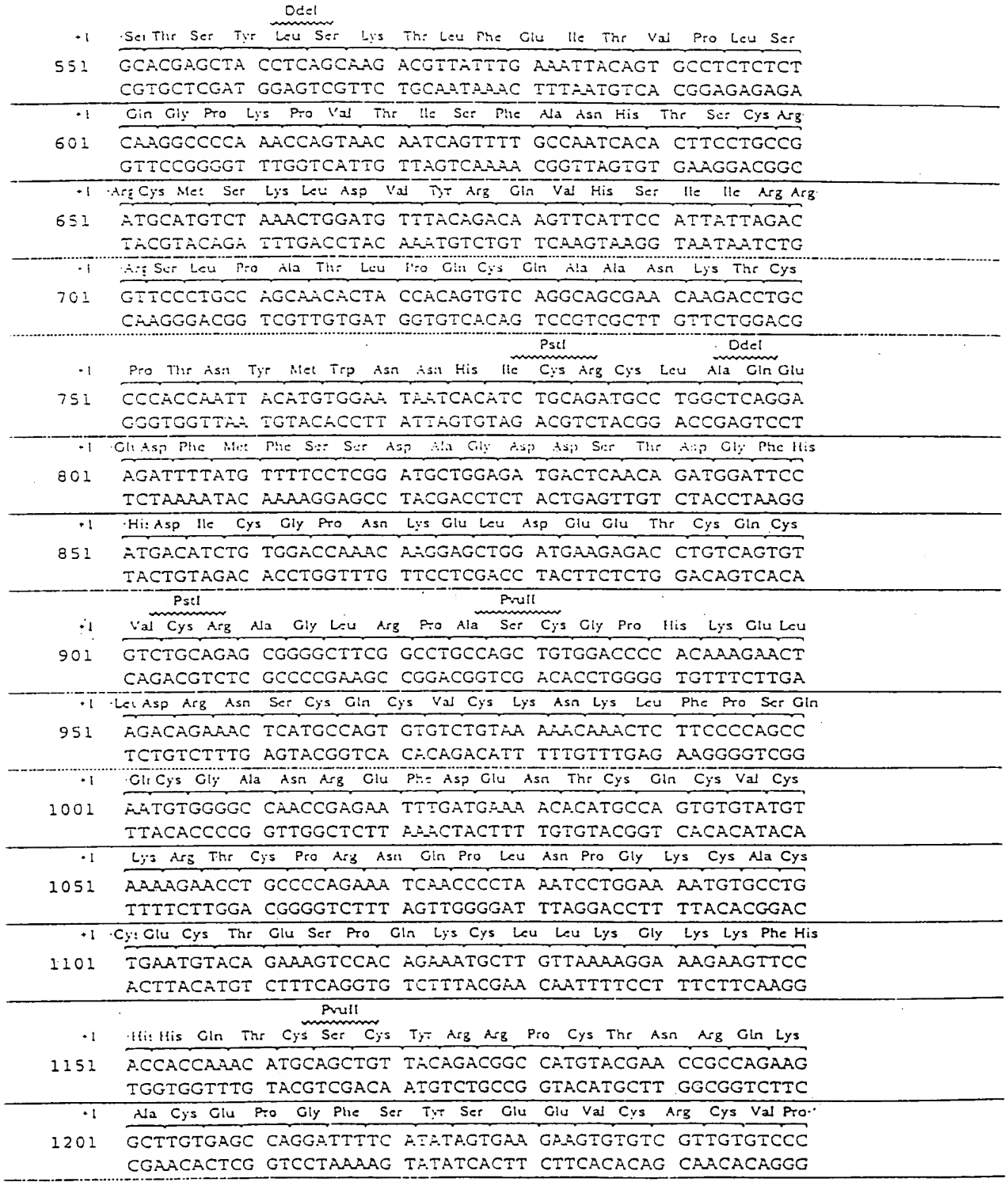
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701 GTTCCCTGCC AGCAACACTA CCACAGTGTC AGGCAGCGAA CAAGACCTGC
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PstI
DdeI
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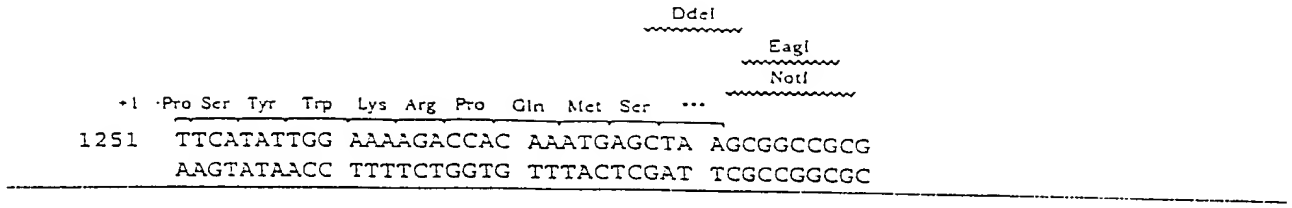

DdeI
EagI
NotI
+1 Pro Ser Tyr Trp Lys Arg Pro Gln Met Ser ...
1251 TTCATATTGG AAAAGACCAC AAATGAGCTA AGCGGCCGCG
AAGTATAACC TTTTCTGGTG TTTACTCGAT TCGCCGGCGC

STRAIN SHEET

STRAIN CMV I

STORAGE NUMBER 11:64

PEDIGREE:

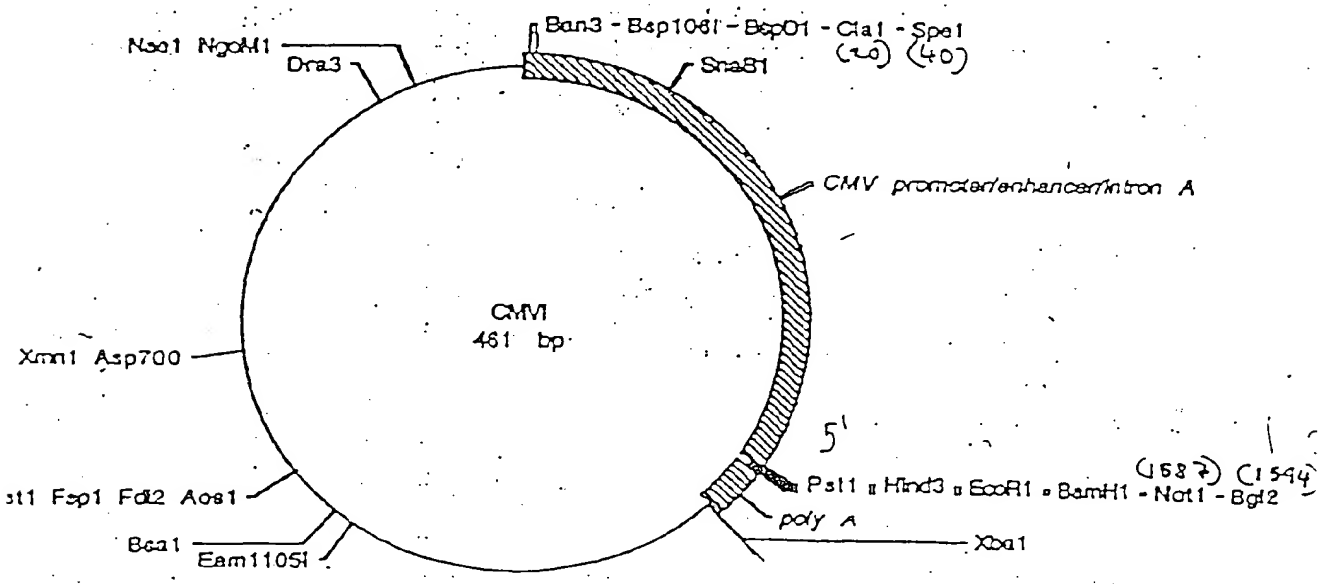
-CMVI was constructed in the pSV7 (nee pSG5 from Stratagene, with an expanded polylinker) backbone by replacing the SV40 promoter from pSV7 with the CMV promoter/enhancer/intron, via 5' Sal I/Xho I (sites destroyed) and 3' Hind III.

-CMVI is 4613 bp.

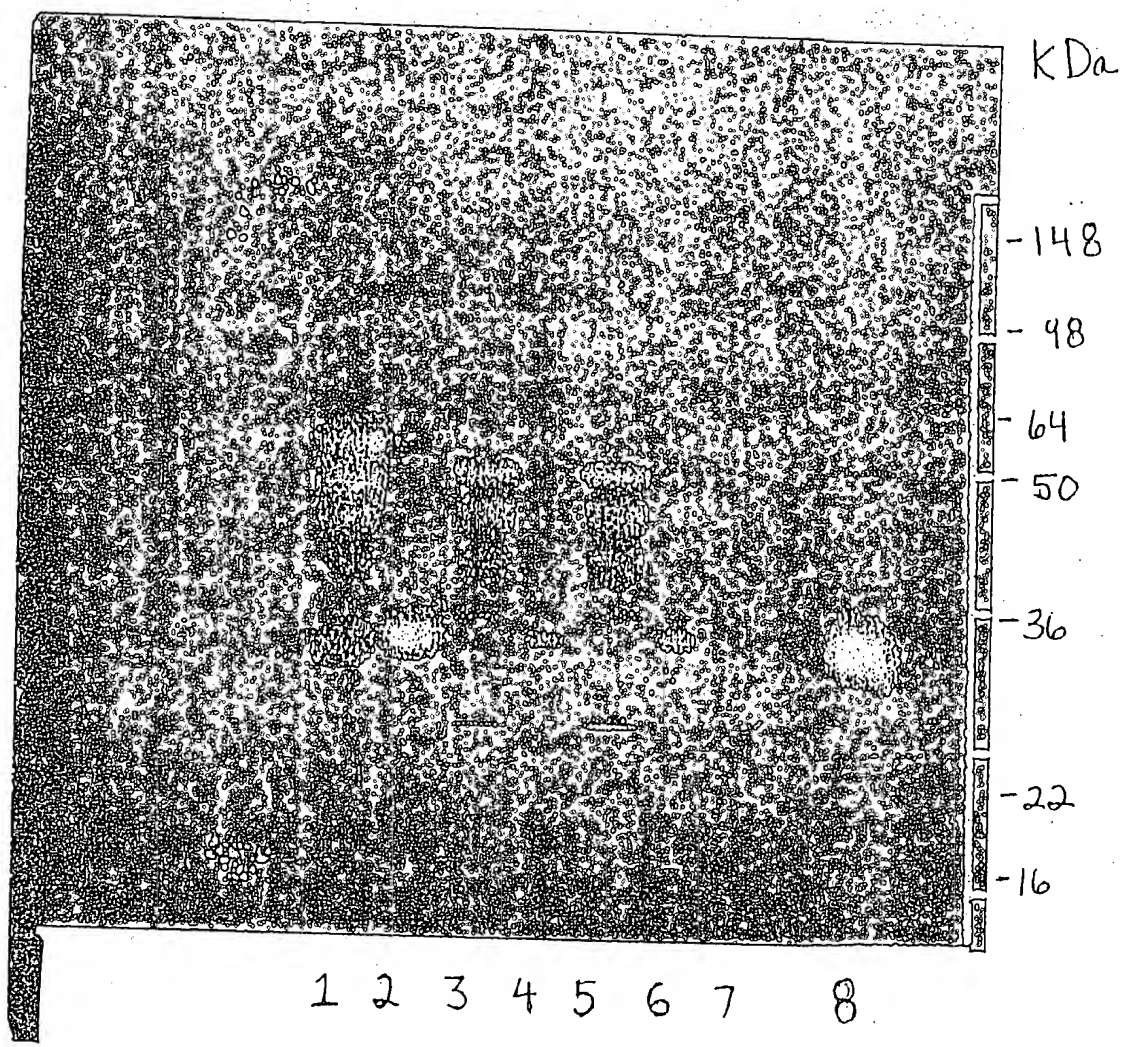
-CMVI uses ampicillin resistance.

-CMV promoter/enhancer + Intron A: nt #'s 1-1566.
-Polylinker: nt #'s 1566-1597 (5'- Hind III - Eco RI - Bam HI - Not I - Bgl II - 3').
-SV40 polyA addition sequence: nt #'s 1598-1745.
-If for some reason you want to remove the SV40 polyA addition sequence, you can cut with either Sal I or Xba I (these 2 sites border the 3' end of this sequence).

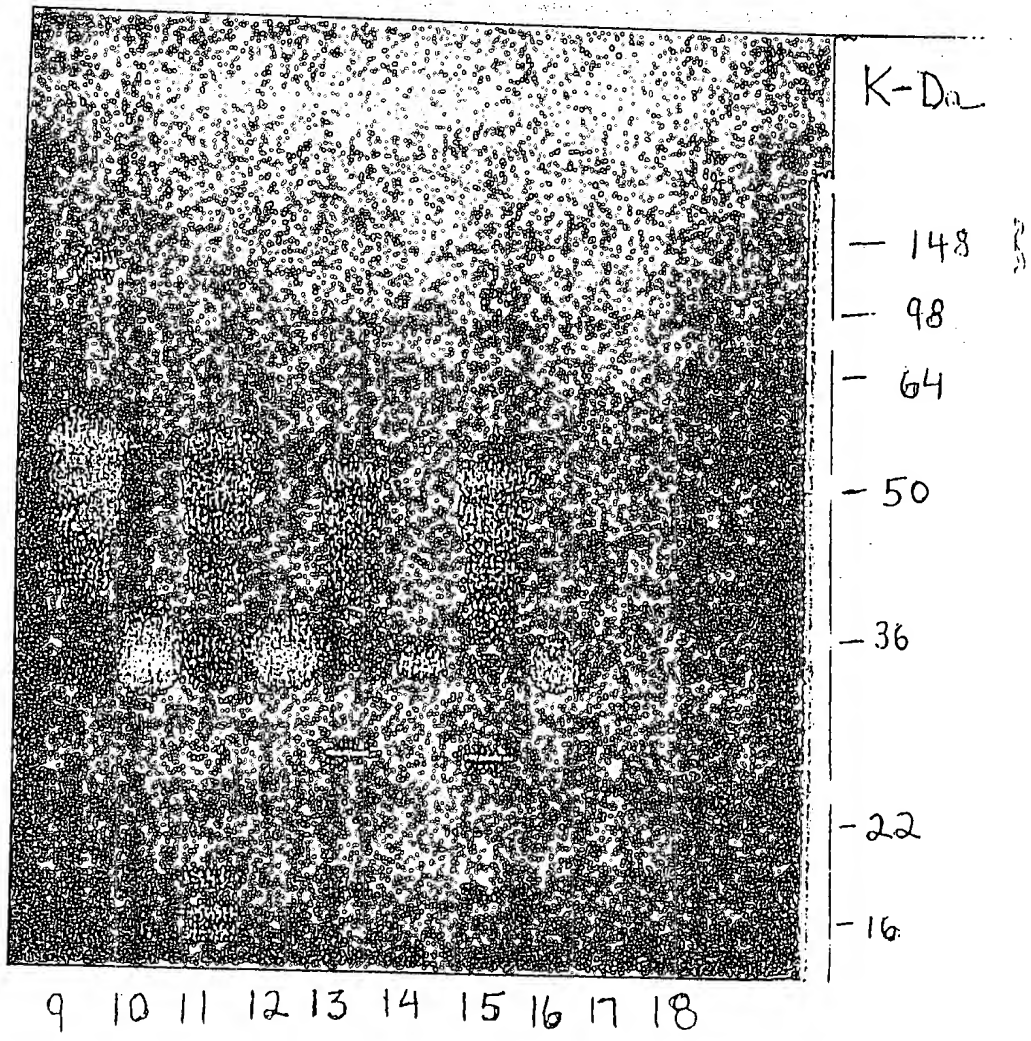
GROWTH REQUIREMENTS:



Appendix II



Power Declaration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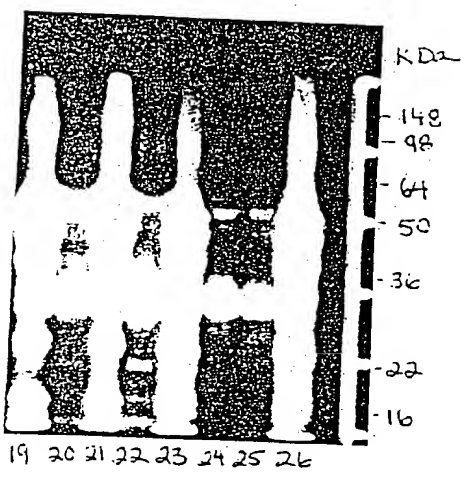

Power Declaration
Figure 1 - Gel 3

Initial Review
BOX AF

esponse Under
37 CFR 1.116- Expedited
Procedure Examining
Group 1814

PATENT
28967/32863

20/c
J.G.J
6/16/97

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Alitalo *et al.*

Serial No: 08/510,133

Filed: August 1, 1995

Title: RECEPTOR LIGAND

Group Art Unit: 1814

Examiner: Lathrop, B.

EXPRESS MAIL LABEL NO: EM099827086US
Date of Deposit: June 11, 1997

I hereby certify that this paper is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

Mark Bonadonna

RECEIVED
JUN 16 1997

AMENDMENT AFTER FINAL ACTION

and

CONDITIONAL PETITION TO REVERSE OR WITHDRAWN ADVERSE PRIORITY DETERMINATION PURSUANT TO 37 C.F.R. §1.181

BOX AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

In an official action mailed April 11, 1997, the examiner finally rejected claims 1, 8, 9, 13-15, and 19-25 variously under 35 U.S.C. §§ 101 and 112, first paragraph. Claims 2 and 12 were allowed, and claims 16 and 17 were objected to as being dependent upon a rejected base claim, but were otherwise deemed allowable. The applicants respectfully request reconsideration in light of the following amendments and remarks.

AMENDMENTS

In the specification:

At page 24, line 30, after "Figure 9" please insert -- (SEQ ID NOs: 32 and 33) --.

In the claims:

Please cancel claims 3-7 and 11, without prejudice, amend claims 8, 16, 17, 21, 22, and 25, and add new claims 26-28, as shown below.

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8. (Three times amended) A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding.

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16. (Amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase and having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions, [according to claim 13,] wherein amino terminal amino acids 2 through 18 of said polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13.

17. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase, [according to claim 1,] said polypeptide being purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase.

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21. (Amended) A polypeptide according to claim [8] 17 further comprising a detectable label.

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22. (Amended) A pharmaceutical composition comprising a polypeptide according to claim [8] 17 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

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25. (Amended) A pharmaceutical composition comprising a polypeptide according to claim [14] 16 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

-- 26. A polypeptide according to claim 8 wherein said portion of SEQ ID NO: 33 effective to permit such binding is a continuous portion of SEQ ID NO: 33 within amino acids 1-180 of SEQ ID NO: 33.

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27. A polypeptide according to claim 8 wherein the amino terminus of said portion effective to permit such binding corresponds with position 34 of SEQ ID NO: 33.

28. A polypeptide according to claim 16 further comprising a detectable label. --

REMARKS

I. History of claims and explanation of amendments.

The application as filed contained twelve claims. In a preliminary amendment (Paper No. 10) dated August 12, 1996, claims 1-4, 6, 8-9, and 12 were amended, and claims 13-19 were added to the application. In an amendment dated February 10, 1997, the applicants canceled claims 10 and 18, amended claims 1, 8, 9, 14, and 17, and added new claims 20-25. In the present amendment, the applicants cancel claims 3-7 and 11, amend claims 8, 16, 17, 21, 22, and 25, and add new claims 26-28. Thus, upon entry of the foregoing amendments, claims 1-2, 8-9, 12-17, and 19-28 would be pending. A copy of the claims in their amended forms is appended hereto.

The nature of each claim amendment is discussed below in the remarks pertaining to each claim.

New claim 26 depends from claim 8 and further limits claim 8 by adopting a suggestion of the examiner with respect to subject matter that the specification enables. Support for the limitation "within amino acids 1-180 of SEQ ID NO: 33" is found in the specification at p. 28, lines 1-3. New claim 27 further limits claim 26 by reciting a specific amino terminal amino acid residue. The particular amino terminus that is recited in claim 27 corresponds to the amino terminus recited in claim 16. This amino terminus finds written support at p. 19, lines 17-19 of the specification.

II. Restriction Requirement

The applicants have canceled non-elected claims 3-7 and 11 without prejudice.

III. **The Applicants respectfully request issuance of an advisory action wherein the Patent Office reverses as incorrect, or withdraws as inappropriate, its determination that no claims in the present application are afforded priority to U.S.S.N. 08/340,011.**

In the outstanding official action, the examiner has asserted, for the first time, that no claims in the present application are entitled to priority based upon U.S.S.N. 08/340,011, filed November 14, 1994, *because of an asserted lack of written description* under 35 U.S.C. §112, first paragraph.[1] For the reasons set forth below, this determination is legally and factually incorrect. Moreover, the right of priority has no bearing on the patentability of any claim at

[1] In its first official action, the examiner made an initial determination that no claims were afforded priority by the '011 application, because of an *asserted absence of enabling disclosure*. However, that initial determination was made without any consideration of the preliminary amendment portion of U.S.S.N. 08/340,011 (a significant omission, since the '011 application is a Rule 62 continuation-in-part of an earlier application, and the preliminary amendment portion of the '011 application is highly pertinent to the priority issue). In the outstanding final action, the priority determination based on lack of enablement has properly been withdrawn. However, the examiner has, for the first time, raised a new objection to the priority claim, based upon an asserted lack of written description.

this time, and therefore, is an inappropriate subject for Patent Office determination.

A. The applicants respectfully request entry into the record and consideration of the expert declaration of Dr. Carl-Henrik Heldin filed herewith.

The Patent Office's reviewing court has explicitly approved of the use of declarations which offer factual evidence to help resolve the issue of "written description" in a patent application, and has held that failure to accord appropriate weight to such declarations constitutes legal error. See *In re Alton*, 37 U.S.P.Q. 1578, 1583 (Fed. Cir. 1996). The applicants have filed herewith the expert declaration of Dr. Carl-Henrik Heldin (the "Heldin declaration") to offer a factual explanation as to *why* one of ordinary skill in the art would have understood the 1994 priority application to describe the invention presently being claimed. Since the examiner raised the written description issue **for the first time** in the outstanding final official action,[2] the applicants respectfully request entry of this declaration into the record and consideration thereof with respect to the issue of written description.

B. The determination that no claims are entitled to priority is legally and factually incorrect.

The law is clear that original claims (i.e., claims contained in the patent application as filed) comply with the written description requirement of §112, because *original claims constitute their own description*. See *In re Koller*, 204 U.S.P.Q. 702, 706 (C.C.P.A. 1980). Moreover, later added claims of similar scope and wording are described by original claims. *Id.*

In the present case, the applicants' 1994 priority application (the '011 application) contained original claims to an Flt4 ligand. For example, original claim 31 recites, "A ligand which specifically binds to an FLT-4 receptor

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[2] The written description issue was <u>not</u> necessitated solely by amendments made by the applicants in response to the first action on the merits, and therefore could have been raised by the Patent Office prior to the issuance of a final action.

tyrosine kinase." By way of comparison, claim 1 of the present application recites, "A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase."

Claim 1 is unquestionably of similar scope and wording to claim 31 as originally filed. Whereas original claim 31 was directed to "a ligand," claim 1 is directed to "a purified and isolated polypeptide." However, the 1994 priority application clearly states that the ligand of the invention is a purified protein. (See, e.g., preliminary amendment to U.S.S.N. 08/340,011 dated November 14, 1994, at p. 15: "The purified biologically active ligand protein . . . .") Whereas original claim 31 was directed to binding to Flt4 receptor tyrosine kinase, claim 1 clarifies that the ligand binds to *the extracellular domain* of Flt4. However, the 1994 priority application clearly states that the ligand protein binds to the Flt4 *extracellular domain*. (See, e.g., preliminary amendment to U.S.S.N. 08/340,011 dated November 14, 1994, at p. 11: "The above experiments prove that the ligand binds to the recombinant FLT4 EC domain.") Finally, original claim 31 recites that the ligand "specifically binds" whereas claim 1 is directed to "high affinity" binding. However, this difference merely adopts preferred claim language suggested by the examiner in the course of prosecution. Thus, claim 1 is unquestionably of similar scope and wording to an original claim of the '011 patent application. (See the Heldin declaration at ¶ 6.) Accordingly, original claims in the '011 patent application provide written description support for claim 1 of the present patent application. See *In re Koller*, 204 U.S.P.Q. at 706.

Claim 19, which depends from claim 1 and recites that the polypeptide further comprises a detectable label, finds written description support in original claim 33 of the 1994 priority application. See *In re Koller*, 204 U.S.P.Q. at 706; see also the Heldin declaration at ¶ 7.

Claim 17 (which depended from claim 1 but has been rewritten as an independent claim incorporating the limitations of claim 1) is similar to claim 1 and additionally recites that the polypeptide is purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, the cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity

purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase. These additional limitations find explicit written description support in the 1994 priority application in Examples 12 and 15: Example 12 teaches that conditioned media from the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) produces a soluble ligand for Flt4 that binds to recombinant Flt4 extracellular domain and that can be purified using the Flt4 EC domain in affinity chromatography; Example 15 describes such affinity chromatography. (See the preliminary amendment to the '011 application at pp. 8-11 and 15.) Thus, claim 17 finds written description support in the original claims of the 1994 priority application coupled with the written description provided in Examples 12 and 15. (See the Heldin declaration at ¶ 8.)

Claim 21 as amended is identical to claim 19 except that claim 21 depends from claim 17. Thus, claim 21 finds written description support in the 1994 priority application for the reasons outlined above with respect to claims 17 and 19.

Claim 14 recites "A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase." Written description support for "a purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase" is discussed above in relation to claim 1. Example 12 in the 1994 priority application teaches that the Flt4 polypeptide ligand stimulates Flt4 tyrosine phosphorylation in mammalian cells that express Flt4 receptor tyrosine kinase. (See the preliminary amendment to the '011 application at pp. 8-11; see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or fragment thereof, which is capable of stimulating the Flt4 receptor . . . .") Thus, claim 14 finds written description support in the original claims of the '011 application coupled with the written description provided in Example 12. (See the Heldin declaration at ¶ 9.)

Claims 13 depends from claim 1 and recites that the polypeptide has an apparent molecular weight of approximately 23 kD as assessed by SDS-

PAGE under reducing conditions. The approximate 23 kD molecular weight is an *inherent property* of the Flt4 ligand that the '011 application teaches one how to purify from the PC-3 conditioned medium. (See the Heldin declaration at ¶¶ 10 and 11.A.) As such, the inclusion of this property in the present application and in claim 13 does not deprive claim 13 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*, 33 U.S.P.Q.2d 1274, 1276 (Fed. Cir. 1995) ("A claim in a CIP application is entitled to the filing date of the parent application when the claimed invention is described in the parent specification in a manner that satisfies, inter alia, the description requirement of 35 U.S.C. §112. . . . [T]he later explicit description of an inherent property does not deprive the product of the benefit of the filing date of the earlier application."); *Ex parte Yamaguchi*, 6 U.S.P.Q.2d 1805, 1807 (PTO Bd. App. 1987) (Claim to compound characterized by a particular x-ray diffraction spectrum has written description support in earlier application that teaches the compound, notwithstanding the absence of any teaching of the x-ray diffraction pattern in the earlier application, because a compound and all of its properties are inseparable); see also *Application of Davies*, 177 U.S.P.Q. 381, 385, 475 F.2d 667, 671-72 (C.C.P.A. 1973) ("[W]e see no impediment to the present applicants' refiling their application and incorporating a discussion of the allegedly unobvious properties [of the invention] while retaining the effective date of the application involved here through §120.")

Claim 15 depends from claim 14 and further recites that the polypeptide "comprises an amino acid sequence set forth in SEQ ID NO: 13." This partial amino acid sequence is an *inherent property* of an Flt4 ligand that the 1994 priority application teaches one how to purify from the PC-3 conditioned medium. (See the present application at p. 19, lines 9-19 (teaching that Flt4 ligand affinity purified from PC-3 medium has an amino terminal amino acid sequence set forth in SEQ ID NO: 13); see also the Heldin declaration at ¶¶ 10 and 11.B.) As such, the inclusion of this property in the present application and in claim 15 does not deprive claim 15 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies*, *supra*.

Claim 16 (which depended from claim 13 but has been rewritten in independent form) additionally recites amino terminal amino acid sequence information of the claimed polypeptide. The recited amino acid sequence is an *inherent property* of the Flt4 ligand that the 1994 priority application teaches one how to purify from PC-3 conditioned medium.[3] (See the Heldin declaration at ¶¶ 10 and 11.C.) As such, the inclusion of this property in the present application and in claim 16 does not deprive claim 16 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies, supra*.

New claim 28 is identical to claim 19 except that claim 28 depends from claim 16. Thus, claim 28 finds written description support in the 1994 priority application for the reasons outlined above with respect to claims 16 and 19.

Claim 23 depends from claim 14 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." This molecular weight limitation is an *inherent property* of an Flt4 ligand that the 1994 priority application teaches one how to purify from the PC-3 conditioned medium, as discussed above with respect to claim 13. (See also the Heldin declaration at ¶¶ 10 and 11.D.) As such, the inclusion of this property in the present application and in claim 23 does not deprive claim 23 of being afforded priority to the '011 application. See *Therma-Tru Corp. v. Peachtree Doors Inc.*; *Ex parte Yamaguchi*; and *Application of Davies, supra*.

Moreover, the foregoing is not intended to be a complete list of those claims which find written description support in the specification. See the Heldin declaration at ¶11.)

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[3] Moreover, the 1994 priority application teaches to determine the amino terminal amino acid sequence. (See preliminary amendment to '011 application at p. 15 (Example 15).)

C. The Patent Office's reliance on the *Fiers* case is improper.

In dismissing the applicants' priority claim on written description grounds, the Patent Office relied upon the Federal Circuit's decision in *Fiers v. Revel*, 25 U.S.P.Q.2d 1601, 1606 (Fed. Cir. 1993). (Official action at pp. 2 and 3.) However, the *Fiers* opinion was rendered on its own distinct set of facts, and was rendered in the context of the state of the art in 1979-81 (i.e., about 13-15 years prior to the applicants' 1994 filing date). Since the issue of written description is factual in nature, *In re Alton*, *supra*, 37 U.S.P.Q.2d at 1580, the examiner's reliance upon a legal opinion rendered on different facts, and in a much earlier period of the art of molecular biology, is highly suspect from the outset.

1. The present application is distinguishable from the facts of the *Fiers* case because the present application teaches a method of preparing the claimed protein as a natural isolate.

The *Fiers* opinion was based on the premise that a written description of a DNA invention requires the same degree of specificity as a conception of a DNA invention. *Fiers*, 25 U.S.P.Q.2d at 1606. Citing its earlier opinion in *Amgen v. Chugai Pharmaceutical Co.*, 18 U.S.P.Q.2d 1016 (Fed. Cir. 1991), the Court acknowledged that conception of a DNA can occur where one is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. *Fiers*, 25 U.S.P.Q.2d at 1604. In the present case, the 1994 priority application is able to define the Flt4 ligand protein by a method of preparation (e.g., affinity purification using the Flt4 extracellular domain) and by chemical characteristics (e.g., a polypeptide that is capable of stimulating the Flt4 receptor and regulating vascular endothelial cells). Thus, under the standards articulated in the *Fiers* and *Amgen* cases for DNA inventions, the 1994 priority application contains a written description of the Flt4 ligand protein invention claimed herein.

2. The present application is distinguishable from *Fiers* because the invention presently claimed pertains to a purified protein.

In *Fiers*, the Federal Circuit rendered an opinion as to that which is required under §112, first paragraph, for an adequate written description of a DNA invention. The invention claimed in the present application is not a DNA invention;[4] the invention pertains to a purified protein, and the issue concerns whether a priority application contains a sufficient written description of that protein invention. The examiner has failed to articulate why a factual determination in *Fiers* pertaining to a DNA invention is relevant to a factual determination pertaining to a protein invention in the present case.[5] Accordingly, the examiner has failed to meet his burden of establishing a *prima facie* case of lack of written description.

Since the *Fiers* holding is distinguishable on its facts and also was rendered in the context of the state of the art in 1979-81, i.e., about 13-15 years prior to the applicants' 1994 filing date, the *Fiers* opinion fails to support the examiner's written description objection.

D. The right of priority has no bearing on the patentability of any claim at this time, and therefore, is an inappropriate subject for Patent Office determination.

The Manual of Patent Examining Procedure instructs that a priority determination should be made during *ex parte* prosecution *only* when an intervening reference is found, upon which a rejection under §102 or §103 would be made:

> The only times during *ex parte* prosecution that the examiner considers the merits of an applicant's claim of

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[4] The Patent Office has deemed the DNAs taught in the application to constitute a distinct invention.

[5] In this regard, the Patent Office's attention is directed to *Scripps Clinic v. Genentech Inc.*, 18 U.S.P.Q. 1001 (Fed, Cir. 1991), an opinion issued contemporaneously with the *Amgen* opinion and pertaining to a purified protein invention. The Patent at issue in the *Scripps* case (Reissue Patent No. 32,011) contained claims to a purified protein (Factor VIII:C) and to an affinity method of purifying the protein. No amino acid sequence description was required under §112, first paragraph, for the Patent Office to issue or to reissue this patent.

> priority is when a reference is found with an effective date between the date of the foreign filing and the date of filing in the United States and when an interference situation is under consideration. If at the time of making an action the examiner has found such an intervening reference, he or she simply rejects whatever claims may be considered unpatentable thereover, without paying any attention to the priority date . . . .

(M.P.E.P.((6th Ed., Rev. 2) §201.15.)

The outstanding final action constitutes the first time that the Patent Office has raised its written description objection as a basis for refusing to afford priority to the '011 application.[6] However, there are no prior art rejections based upon intervening references in the outstanding action. Accordingly, under the Patent Office's own procedures, it was inappropriate to consider the merits of the priority claim in the official action.

E. Conditional Petition to Reverse or Withdrawn Adverse Priority Determination.

Should the examiner refuse to reverse or withdraw the adverse priority determination that was made for the first time in the final official action, the applicants hereby petition the commissioner to reverse this determination as improper, or, in the alternative, to withdraw this determination as premature and expunge from the file all mention of this premature determination. The facts in support of reversal of the priority determination are provided in parts A-C, above, and in the Declaration of Dr. Heldin filed herewith. The facts in support of withdrawal of the premature determination are provided in part D, above. In the event of withdrawal, the applicants respectfully submit that all mention of the priority determination in the final official action and this submission by the applicants should be expunged from the file, so as not to taint the file history of the eventual patent in a manner adverse to the applicants.

The priority issue is properly the subject of a petition because the priority determination is not pertinent to any rejection and, therefore, is not

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[6] See note 1, *supra*.

subject to review by the Board of Patent Appeals and Interferences. See M.P.E.P. §706.01.

The applicants hereby authorize the commissioner to charge any necessary petition fee associated with this conditional petition to Deposit Account No. 13-2855. This petition has been timely filed within two months of the mailing of the final official action that contains the adverse priority determination at issue.

IV. The amendments to claim 8 render moot the rejection of claims 8-9 and 19-20.

In paragraph 9 of the outstanding official action, the examiner rejected claims 8-9 and 20-22 under 35 U.S.C. § 101, asserting that these claims read on a product of nature, because claim 8 fails to recite a "purified and isolated" polypeptide. (Office action at p. 5.)

In response, the applicants have amended claim 8 to recite, "A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding." Thus, amended claim 8 does not read on a product of nature, rendering the rejection of claim 8 (and claims 9 and 19-20 which depend therefrom) moot. Since this amendment adopts a suggestion of the Patent Office and removes an issue for appeal, entry of the amendment and withdrawal of the rejection is respectfully requested.

V. The amendments to claims 16 and 17 place these claims in condition for allowance.

In paragraph 15 of the outstanding action, the Patent Office objected to claims 16 and 17 as being dependent upon a rejected base claim, but indicated that these claims would be allowable if rewritten in independent form. (Office action at p. 11.) In response, the applicants have rewritten claims 16 and 17 in independent form, incorporating all of the limitation of the base claim and any intervening claims. Accordingly, claim 16 and 17 are now in condition for allowance.

VI. **The amendments to claims 21, 22, and 25 place these claims in condition for allowance; and new claim 28 is in condition for allowance.**

Claims 21, 22, and 25 have been amended to depend from and further limit claims 16 and 17. New claim 28 is identical to claim 21 and depends from claim 16. Because the subject matter of claims 16 and 17 has been deemed allowable, the amendment of claims 21, 22, and 25 (and addition of claim 28) to depend from claims 16 and 17 also places these claims in condition for allowance. Accordingly, entry of these amendments and allowance of claims 21, 22, 25, and 28 is respectfully requested.

VII. **The Patent Office's rejections of clams 1, 8, 9, 13-15, and 19-25 under §112, first paragraph, for lack of enablement improperly ignore both guidance provided in the specification and the skill of those in the art.**

In paragraphs 10-13 of the official action, the examiner articulated his basis for maintaining rejections of claims 1, 8, 9, 13-15, and 19-25 under §112, first paragraph, for lack of enablement. The Patent Office admits that fragments of the protein of SEQ ID NO: 33 can be made, but asserts that undue experimentation would be required to screen **all** fragments of SEQ ID NO: 33 to determine which fragments bind the receptor:

> The examiner argues that the claim limitation of binding the receptor must be met by testing all fragments encompassed by the claims, which in this case are not limited in any way.

(official action at p. 7.)[7]

The Patent Office's insistence that it is necessary to test **all** fragments of SEQ ID NO: 33 ignores the scientific ability of one of ordinary skill in the art. Importantly, one of ordinary skill in the art would not conduct experimentation by haphazardly making <u>all</u> of the possible fragments of SEQ ID NO: 33 and testing their ability to bind the receptor. An artisan of ordinary skill understands that each fragment that is screened provides guidance as to that

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[7] Claim 8 encompasses only polypeptides which are capable of binding the Flt4 receptor. To the extent that the examiner has interpreted claim 8 (or similarly limited claims) to "encompass" **all** fragments of SEQ ID NO: 33, the examiner has ignored a limitation of claim 8 and thereby erroneously construed the claim.

portion of SEQ ID NO: 33 that is effective for binding, and that portion which is not.[8] An artisan of ordinary skill also understands techniques for accelerating a screening process,[9] and techniques for screening multiple polypeptides *simultaneously*. Thus, the examiner's reasoning greatly overstates both the quantity and the nature of the experimentation required to practice the invention as claimed.

In this regard, the application provides explicit guidance for screening fragments of SEQ ID NO: 33 to determine a portion effective to permit Flt4 binding. Although SEQ ID NO: 33 contains 350 amino acids, the specification provides guidance that the region critical for receptor activation is contained **within** its first approximately 180 amino acid residues.

> By extrapolation from studies of the structure of the related platelet derived growth factor (PDGF, reference Heldin, et al., Growth Factors 8, 245-252, 1993) one determines that the region critical for receptor activation by the Flt4 ligand is contained within its first approximately 180 amino acid residues. (Specification, pp. 27-28.)

To determine which fragments contain a sufficient portion of SEQ ID NO: 33 to permit binding, the specification also outlines a specific protocol. The specification teaches one skilled in the art to (a) generate progressive deletion products of the Flt4 ligand cDNA; (b) express these modified cDNAs; and (c) assay the resulting truncated protein forms, e.g., by studying their ability to induce Flt4 autophosphorylation. (Specification at, e.g., p. 27, lines 23-29.) These teachings serve to both provide guidance for predicting the portions of

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[8] For example, a determination that a polypeptide comprising residues 34-180 of SEQ ID NO: 33 is effective to permit binding to Flt4 and that a polypeptide comprising residues 181-350 is ineffective to permit binding would provide significant guidance as to that portion of SEQ ID NO: 33 to further screen for effective fragments. Thus, the assertion that it would be necessary to screen "all" fragments of SEQ ID NO: 33 to practice the claimed invention relies upon the false assumption that individual screening assays will be performed without knowledge gained from prior screenings.

[9] For example, it is within the skill of the art to synthesize spaced deletion mutants (e.g., residues 34-350, 34-330, 34-310, etc.) from SEQ ID NO: 33, rather than successive deletion mutants (34-350, 34-349, 34-348 . . .), to more rapidly identify effective portions for binding Flt4.

SEQ ID NO: 33 that are effective to permit Flt4 binding; and (2) reduce the amount of experimentation required to determine the minimum portion of SEQ ID NO: 33 that is critical for receptor binding.

Moreover, as explained above, it is within the skill of the art to synthesize deletion mutants of SEQ ID NO: 33 that have been spaced intermittently (e.g., residues 34-180, 34-160, 34-140, 34-120, etc.), rather than synthesize every possible successive deletion mutant (34-180, 34-179, 34-178, 34-177 . . .), to more rapidly identify effective portions for binding Flt4. Furthermore, the skilled artisan is capable of synthesizing and screening several such deletion fragments simultaneously, in parallel experiments. Thus, the examiner's assertions that it is necessary to screen every fragment of SEQ ID NO: 33, that the specification lacks guidance, and that the amount of screening required constitutes undue experimentation is improper. See *In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988) ("Enablement is not precluded by the necessity for some experimentation such as routine screening. . . . The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.")

VIII. Summary

For the foregoing reasons, the applicants respectfully request reconsideration, withdrawal of all claim rejections and objections to the specification, withdrawal of the notation that no claims are afforded priority to the parent application, and allowance of claims 1-2, 8-9, 12-17, and 19-28.

Respectfully submitted,

June 11, 1997

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Appendix of claims

1. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase.

2. (Amended) A purified and isolated polypeptide comprising an amino acid sequence shown in SEQ ID NO: 33.

8. (Three times amended) A purified and isolated polypeptide capable of binding to an Flt4 receptor tyrosine kinase, said polypeptide comprising a portion of SEQ ID NO: 33 effective to permit such binding.

9. (Twice amended) A polypeptide according to claim 8 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

12. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 2 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

13. A polypeptide according to claim 1 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

14. (Amended) A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

15. A purified and isolated polypeptide according to claim 14, said polypeptide comprising an amino acid sequence set forth in SEQ ID NO: 13.

16. (Amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase and having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions, wherein amino terminal amino acids 2 through 18 of said polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13.

17. (Twice amended) A purified and isolated polypeptide capable of binding with high affinity to the extracellular domain of Flt4 receptor tyrosine kinase, said polypeptide being purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase.

19. A polypeptide according to claim 1 further comprising a detectable label.

20. A polypeptide according to claim 8 which is capable of binding the extracellular domain of Flt4 receptor tyrosine kinase with high affinity and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

21. (Amended) A polypeptide according to claim 17 further comprising a detectable label.

22. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 17 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

23. A polypeptide according to claim 14 having an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions.

24. A polypeptide according to claim 14 comprising a portion of SEQ ID NO: 33 effective to permit binding to Flt4 receptor tyrosine kinase and stimulation of Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase.

25. (Amended) A pharmaceutical composition comprising a polypeptide according to claim 16 in a pharmaceutically-acceptable diluent, adjuvant, or carrier.

26. A polypeptide according to claim 8 wherein said portion of SEQ ID NO: 33 effective to permit such binding is a continuous portion of SEQ ID NO: 33 within amino acids 1-180 of SEQ ID NO: 33.

27. A polypeptide according to claim 8 wherein the amino terminus of said portion effective to permit such binding corresponds with position 34 of SEQ ID NO: 33.

28. A polypeptide according to claim 16 further comprising a detectable label.

PATENT
28967/32863

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Application of:

Alitalo et al.

Serial No.: 08/510,133

Filed: August 1, 1995

For: RECEPTOR LIGAND

Group Art Unit: 1814

Examiner: Lathrop, B.

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Date of Deposit: June 11, 1997

I hereby certify that this paper and the documents referred to as enclosed herewith are being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR §1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Mark Bonadonna

Declaration of Carl-Henrik Heldin
Pursuant to 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Carl-Henrik Heldin, hereby state as follows:

1. I am Director and member of the Uppsala Branch of Growth Regulation of the Ludwig Institute of Cancer Research (the Ludwig Institute) in Uppsala, Sweden. My curriculum vitae is attached hereto as Exhibit A.

2. I understand that on 01 August 1995, Dr. Kari Alitalo and Dr. Vladimir Joukov (as inventors) filed U.S. Patent Application Serial No. 08/510,133 (hereinafter "the 1995 application"), directed to a polypeptide ligand for Flt4 receptor tyrosine kinase; fragments thereof; a polynucleotide encoding the ligand; vectors and host cells comprising the polynucleotide; and

antibodies reactive with the ligand. I understand that the Ludwig Institute now has an ownership interest in this application.

3. I further understand that, during examination of the 1995 application by the U.S. Patent and Trademark Office (the Patent Office), the examiner has taken the position that U.S. Patent Application Serial No. 08/340,011, filed on 14 November 1994 ("the 1994 application") does not contain a written description of the polypeptide invention that is being claimed in the 1995 application. I have been asked by the Ludwig Institute to review the 1994 and 1995 applications and to provide a factual analysis of whether the 1994 application contains a written description of the invention that is being claimed in the 1995 application.

4. I understand that the claims in a patent application are the portion of a patent application that defines the invention for which patent applicants seek patent protection. I further understand that patent applications are written for the practitioner of ordinary skill in the pertinent scientific field. In the scientific specialties or subdisciplines which fall within the general category of "cellular and molecular biology," the reader of ordinary skill in 1994 and 1995 (hereinafter "the reader") would have had at least a medical or doctorate degree and probably at least some post-doctoral research experience.

5. To perform this analysis, I have reviewed and understand the contents of the 1994 application. This review included the document titled "Preliminary Amendment" that was filed on 14 November 1994 (hereinafter "the Preliminary Amendment"). I understand that pages 2-19 of the Preliminary Amendment contain text, examples, and claims which are considered part of the 1994 application. I also have reviewed and understand the contents of the 1995 application, including the claims thereof. Exhibit B hereto contains the pending claims of the 1995 application, with claim amendments that the Applicants intend to file with the Patent Office contemporaneously with this declaration.

6. From the facts summarized below, I conclude that the subject matter of claim 1 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Stated another way, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 1 of the 1995 application, at the time that the 1994 application was filed:

A. Claim 31 of the 1994 application recites, "A ligand which specifically binds *to an FLT-4 receptor tyrosine kinase*." (See the Preliminary Amendment at p. 18.) Since claims in a patent application define the invention for which patent applicants seek patent protection, it is absolutely clear to me that the inventors considered an Flt4 ligand to be an aspect of their invention.

B. Claim 1 of the 1995 application recites, "A purified and isolated polypeptide capable of binding with high affinity *to the extracellular domain of Flt4 receptor tyrosine kinase*." Thus, whereas claim 31 of the 1994 application was directed to "a ligand," claim 1 of the 1995 application is directed to "a purified and isolated polypeptide." However, the 1994 application clearly states that the ligand of the invention is a purified protein. (See, e.g., the Preliminary Amendment at p. 15 ("The purified biologically active ligand protein . . . ."); see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or a fragment thereof, which is capable of stimulating the FLT4 receptor....").) Therefore, the "purified and isolated polypeptide" recitations of claim 1 are described in the 1994 application.

C. Whereas claim 31 of the 1994 application was directed to binding "to an FLT-4 receptor tyrosine kinase," claim 1 of the 1995 application specifies that the ligand binds "to *the extracellular domain* of Flt4 receptor tyrosine kinase." However, the 1994 application clearly states that the ligand protein binds to the Flt4 *extracellular domain*. (See, e.g., the Preliminary Amendment at p. 11: "The above experiments prove that the ligand binds to the recombinant FLT4 EC [extracellular] domain.")

Therefore, the recitations in claim 1 regarding binding to the Flt4 *extracellular domain* are described in the 1994 application.

D. Claim 31 of the 1994 application recites that the ligand "specifically binds," whereas claim 1 of the 1995 application is directed to "high affinity" binding. However, the reader would have understood that the "ligand" that "specifically binds" to Flt4 receptor was a high affinity binding partner. For example, the teaching in the 1994 application to purify the ligand using the recombinant FLT4 EC domain in affinity chromatography (see, e.g., the Preliminary Amendment at p. 11 and Example 15) apprises the reader that the ligand is thought to be a high affinity ligand.

Thus, I conclude that the subject matter of claim 1 of the 1995 application is described in claim 31, at pp. 11 and 15 of the Preliminary Amendment, and elsewhere in the 1994 application.

7. I conclude that the subject matter of claim 19 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 19 of the 1995 application is directed to the polypeptide having all of the features recited in claim 1 of the 1995 application, and "further comprising a detectable label." Thus, the only aspect of claim 19 not already discussed above (in paragraph 6) is the inclusion of a detectable label. However, claim 33 of the 1994 patent application recites, "The ligand according to claim 31 comprising a label." (See the Preliminary Amendment at p. 18.) Since claims in a patent application define the invention for which patent applicants seek patent protection, it is absolutely clear to the reader from claims 31 and 33 of the 1994 application that the inventors considered an Flt4 ligand which includes a label to be an aspect of their invention. The property of being "detectable" is understood in the art to be inherent in a "label." (The purpose of a label is to provide a means for detecting the substance that carries the label.) Moreover, this understanding is confirmed by claims 34 and 35 of the 1994 application, which are directed to methods which involve "detecting" the labeled ligand.

(See the Preliminary Amendment at p. 19.) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 19 of the 1995 application, at the time that the 1994 application was filed.

8. I conclude that the subject matter of claim 17 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 17 is similar to claim 1 of the 1995 application and additionally recites that the polypeptide is "**purifyable from conditioned media from a PC-3 prostatic adenocarcinoma cell line, said cell line having ATCC CRL No. 1435, using an affinity purification procedure wherein the affinity purification matrix comprises a polypeptide comprising the extracellular domain of Flt4 receptor tyrosine kinase.**" These additional properties are explicitly described in the 1994 application in Examples 12 and 15: Example 12 teaches that conditioned media from the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) produces a soluble ligand for Flt4 that binds to recombinant Flt4 extracellular domain and that can be purified using the Flt4 EC domain in affinity chromatography (see the Preliminary Amendment at pp. 8-11); Example 15 describes such affinity chromatography. (*Id.* at p. 15.) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 17 of the 1995 application, at the time that the 1994 application was filed.

9. I conclude that the subject matter of claim 14 of the 1995 application is described in the 1994 application in a manner which apprises the reader that the inventors had possession of a concept of what is claimed. Claim 14 recites, "A purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase and stimulating Flt4 phosphorylation in mammalian cells expressing Flt4 receptor tyrosine kinase." Descriptive support in the 1994 application for "a purified and isolated polypeptide which is capable of binding to Flt4 receptor tyrosine kinase" is discussed above with respect to claim 1. (See paragraph 6, above.) Example 12 in the 1994 application teaches that the

Flt4 polypeptide ligand stimulates Flt4 tyrosine phosphorylation in mammalian cells that express Flt4 receptor tyrosine kinase. (See the Preliminary Amendment at pp. 8-11; see also p. 6 ("In a preferred embodiment of the invention, conditioned medium from the PC-3 cell line comprises a protein or fragment thereof, which is capable of stimulating the Flt4 receptor....").) Thus, the 1994 application reasonably conveys to me that the inventors had possession of the subject matter of claim 14 of the 1995 application, at the time that the 1994 application was filed.

10. It is fundamental biochemistry that polypeptides are organic chemical compounds, albeit sometimes large and complex ones. Like all organic chemical compounds, polypeptides may be characterized by any of several inherent physical properties, such as molecular formula and molecular weight. Such physical properties are *inherent* characteristics of organic molecules in that they are intrinsic properties of the molecules. Because polypeptides are themselves composed of covalently-bonded chains of smaller organic moieties called amino acids (of which there are about 20 naturally occurring), it is conventional to express the molecular formula of polypeptides as an amino acid sequence. The amino acid sequence of any polypeptide is an inherent property of that polypeptide.

11. Certain claims in the 1995 application recite subject matter that is described in the 1994 application, and also recite certain inherent properties of that subject matter.

A. For example, claims 13 recites a polypeptide having all of the characteristics described in claim 1 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." The subject matter of claim 1 is described in the 1994 application. (See paragraph 6, above.) The approximate 23 kD molecular weight that is recited in claim 13 is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from the PC-3 conditioned medium. (See the

1995 application at pp. 18-19 (teaching that the Flt4 ligand that was affinity purified from PC-3 medium had an apparent molecular weight of about 23 kD as assessed by SDS-PAGE under reducing conditions).)

B. Claim 15 recites a polypeptide having all of the characteristics described in claim 14 and further recites that the polypeptide comprises "an amino acid sequence set forth in SEQ ID NO: 13." The partial amino acid sequence set forth in SEQ ID NO: 13 of the 1995 application is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from the PC-3 conditioned medium. (See the 1995 application at p. 19, lines 9-19 (teaching that Flt4 ligand that was affinity purified from PC-3 medium had an amino terminal amino acid sequence set forth in SEQ ID NO: 13).)

C. Claim 16 recites a polypeptide having all of the characteristics described in claim 13 and further recites that amino acids 2 through 18 of the polypeptide have an amino acid sequence corresponding to amino acids 2 through 18 set forth in SEQ ID NO: 13. Thus, for the reasons described above with respect to claims 13 and 15 (in Parts A and B), the features recited in claim 16 are inherent properties of an Flt4 ligand that the 1994 application teaches one how to purify from PC-3 conditioned medium.

D. Claim 23 recites a polypeptide having all of the characteristics described in claim 14 and further recites that the polypeptide has "an apparent molecular weight of approximately 23 kD as assessed by SDS-PAGE under reducing conditions." The subject matter of claim 14 is described in the 1994 application. (See paragraph 9, above.) The approximate 23 kD molecular weight further recited in claim 23 is an *inherent property* of an Flt4 ligand that the 1994 application teaches one how to purify from a PC-3 conditioned medium, as discussed in Part A above with respect to claim 13.

The foregoing is not intended to constitute a complete list of those claims which recite inherent properties of an Flt4 ligand described in the 1994 application. For example, the 1995 application teaches a cDNA nucleotide sequence and a

deduced amino acid sequence of a precursor of a 23 kD Flt4 ligand taught in the 1994 application. (See, e.g., 1995 application at p. 5, lines 13-20.) Thus, according to the 1995 application, an inherent property of an Flt4 ligand taught in the 1994 application is that the ligand has an amino acid sequence comprising a portion of SEQ ID NO: 33 that is effective to permit binding to Flt4 receptor tyrosine kinase and stimulate phosphorylation thereof. These properties are recited in several claims of the 1995 application other than those specifically discussed above.

12. The 1994 application teaches the reader how to purify and isolate an Flt4 ligand from conditioned medium of a prostatic cell line, using an affinity chromatography method:

A. Example 12 in the 1994 application teaches the reader how to prepare a conditioned medium comprising an Flt4 ligand by culturing the PC-3 prostatic adenocarcinoma cell line (ATCC CRL 1435) for seven days in F12 medium in the absence of serum, and then clarifying the medium by centrifugation. (See the Preliminary amendment at p. 8.) Example 4 in the 1995 application contains a similar teaching.

B. Example 12 in the 1994 application contains experimental data proving that the PC-3 conditioned medium contains a ligand that is capable of stimulating tyrosine phosphorylation of Flt4 receptor tyrosine kinase, in cells expressing Flt4 receptor tyrosine kinase. (See the Preliminary Amendment at pp. 8-11.) Moreover, Example 12 in the 1994 application characterizes the Flt4 ligand as a moiety of at least 10,000 molecular weight, and teaches that the medium can be concentrated with a commercially available Centricon-10 concentrator, in order to increase Flt4 ligand activity. (Preliminary Amendment at p. 11.)

C. Example 12 further teaches that treatment of the concentrated PC-3 conditioned medium with Flt4 extracellular domain fragment coupled to Sepharose beads (a solid support) will remove the Flt4 ligand from the conditioned medium. (See the Preliminary Amendment at p. 11 (pretreatment of the concentrated conditioned medium with Flt4EC-

Sepharose abolished the ability of the conditioned medium to stimulate Flt4 phosphorylation).) This teaching provides direct evidence that the ligand of the invention binds to the extracellular domain of Flt4, and thus that the ligand can be purified using the recombinant Flt4 extracellular domain in affinity chromatography.

D. Example 14 of the 1994 application teaches how to make recombinant Flt4 extracellular domain protein to use in an affinity chromatography matrix to purify the Flt4 ligand. (See, e.g., the Preliminary Amendment at p. 13.) Example 3 of the 1995 application contains a similar teaching.

E. Example 15 of the 1994 application teaches how to purify the Flt4 ligand using affinity chromatography procedures. In one of the procedures, the affinity matrix is Flt4 extracellular domain protein that has been cross-linked to CNBr-activated Sepharose 4B (a commercially available solid support that is useful for generating an affinity matrix). The reader in 1994 would have understood that affinity purification involves contacting the ligand-containing solution with the affinity matrix to permit binding between the ligand and the affinity matrix; washing the affinity matrix to remove unbound impurities; and eluting the ligand with an eluting solution. Typically, all fractions removed from the matrix (wash fractions and elution fractions) are assayed to determine in which fractions the ligand of interest has eluted. Example 15 of the 1994 application teaches to use an Flt4 phosphorylation assay to determine which chromatography fractions contained the Flt4 ligand. (See the Preliminary Amendment at p. 15.) The phosphate buffered saline and phosphate buffer wash solutions that were actually used (see the 1995 application at Example 5, p. 18) are typical wash solutions for a protein affinity chromatography. Moreover, the reader would have known that varying parameters such as ionic strength, pH, and the hydrophilic/hydrophobic character of the eluting solutions are conventional methods for eluting a compound of interest from an affinity chromatography column. Thus, the details in Example 15 of the 1994

application enable the reader to purify the Flt4 ligand by affinity chromatography.

F. The 1994 application teaches to subject the Flt4 ligand material that is eluted from the affinity column to further purification, using ion exchange and reverse-phase high pressure chromatography and SDS-polyacrylamide gel electrophoresis. (See the Preliminary Amendment at p. 15.) While the reader would have been able to perform all three of these conventional techniques, it is clear from the results reported in the 1995 application that sufficiently pure Flt4 ligand is obtained (e.g., sufficiently pure for amino acid sequencing) simply with the affinity purification followed by the SDS-PAGE procedure. (See the 1995 application at Example 5, pp. 17-19.) The ion exchange and reverse-phase chromatography were unnecessary.

Thus, the 1994 application teaches the reader how to purify and isolate an Flt4 ligand. The 1995 application describes results of such a purification, thereby demonstrating that the affinity purification method taught in the 1994 application works successfully.

13. The 1994 application teaches several uses for purified Flt4 ligand. These uses include:

A. Isolating a gene encoding the Flt4 ligand by microsequencing the purified ligand to determine a partial amino acid sequence; generating oligonucleotide probes based on the amino acid sequence (See the Preliminary Amendment, Example 15, p. 15; and Example 12, pp. 11-12); using the oligonucleotides as hybridization probes or PCR primers to isolate a ligand-encoding cDNA clone from a cDNA library generated from PC-3 poly-A RNA (*Id.*, Examples 16 and 17A, p. 16);

B. use in an assay system to screen for inhibitors of Flt4 ligand/Flt4 receptor tyrosine kinase interaction (Preliminary Amendment at pp. 6 and 7);

C. regulating the growth, differentiation, and functions of endothelial cells, particularly lymphatic endothelia (Preliminary Amendment at p. 7);

D. generating antibodies against the Flt4 ligand (Preliminary Amendment at p. 7);

E. use in an assay to detect the presence of FLT4 receptor tyrosine kinase (see the Preliminary Amendment at p. 19, claim 35); and

F. use in an assay to detect endothelial cell proliferation (*Id.* , claim 34).

14. With respect to my conclusions in paragraphs 6-13, above, I believe that the reader of ordinary skill in the field in 1994 who reviewed the 1994 application would have reached the same conclusions: that the inventors had possession of a concept of what is now being claimed in the present application. Stated another way, the priority application reasonably would have conveyed to the skilled artisan that the inventors had possession of the Flt4 ligand invention recited in claims of the 1995 application, of how to purify the ligand, and how to use the ligand.

15. I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

June 4, 1999
Date

Carl-Henrik Heldin

CURRICULUM ITAE 97-05-28

Name:		Carl-Henrik Heldin
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Home address:		Hesselmans väg 35 S-752 63 Uppsala, Sweden Phone: +46-18-463213
Date and place of birth:		August 9, 1952, Växjö, Sweden
Nationality:		Swedish
Marital status:		Married, two children, born 1982 and 1988
University education:	1971-1975	First four years of Medical School completed (University of Uppsala)
	1972-1981	Bachelor of Science (Mathematics 1 1/2 year, Numeric analysis 1/2 year, Psychology 1/2 year, Greek 1/2 year) completed July 28, 1981 (University of Uppsala)
	1975-1980	Thesis work at Department of Medical and Physiological Chemistry (University of Uppsala). Dissertation May 10, 1980. "Studies on growth factors for human cultured cells".
Academic positions:	1972-1974	Part time teaching positions at Depts of Anatomy, Medical and Physiological Chemistry, and Physiology (in total 200 hours)
	75.07.01- 80.03.31.	Graduate student scholarship at Dept of Medical and Physiological Chemistry combined with a part time teaching position (in total 1100 hours)
	80.04.01- 80.10.31	Research Assistant at Dept of Medical and Physiological Chemistry
	81.01.01- 81.03.31	Lecturer at Dept of Medical and Physiological Chemistry
	81.07.01- 83.12.31	Cancer Research Scholarship from the Swedish Cancer Society
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	86.01.01--	Director, Ludwig Institute for Cancer Research (Uppsala Branch)
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